Crystal structure of the dimeric unswapped form of bovine seminal ribonuclease

R. Berisio[a], F. Sica[a,b], C. De Lorenzo[c], A. Di Fiore[b], R. Piccoli[c,d], A. Zagari[a,c], L. Mazzarella[a,b,*]

[a] *Istituto di Biostrutture e Bioimmagini, CNR, Via Mezzocannone 6, 80134 Naples, Italy*
[b] *Dipartimento di Chimica, Università di Napoli Federico II, Via Cynthia, 80126 Naples, Italy*
[c] *Dipartimento di Chimica Biologica, Università di Napoli Federico II, Via Mezzocannone 16, 80134 Naples, Italy*
[d] *Facoltà di Scienze Biotecnologiche, Università di Napoli Federico II, Naples, Italy*

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Abstract **Bovine seminal ribonuclease is a unique case of protein dimorphism, since it exists in two dimeric forms, with different biological and kinetic behavior, which interconvert into one another through three-dimensional swapping. Here we report the crystal structure, at 2.2 Å resolution, of the unswapped form of bovine seminal ribonuclease. Besides completing the structural definition of bovine seminal ribonuclease conformational dimorphism, this study provides the structural basis to explain the dependence of the enzyme cooperative effects on its swapping state.**

Key words: Three-dimensional domain swapping; Conformational dimorphism; Crystal structure; Ribonuclease

1. Introduction

It is commonly believed that proteins are evolutionarily shaped to perform their specific tasks. Protein polymerization into multimeric structures seems to be controlled, at least in part, by their tendency to adopt alternative conformations (conformational polymorphism). The capability of some proteins to establish stable intermolecular interactions through the reciprocal exchange of structural elements by identical protomers, a phenomenon known as 3D-domain swapping, has been proposed as a possible mechanism of protein evolution from monomeric to oligomeric states [1,2]. Over 40 swapping proteins have been described so far [3].

Biological interest in 3D-domain swapping has further increased in recent years, since it has been proposed to be a possible mechanism for amyloid fiber formation [4]. This hypothesis has been supported by the observation that both human prion protein [5] and cystatin C [6,7], which form fibers and are related to amyloid diseases [8], are domain-swapped.

In almost all of the known swapping proteins either the N-terminal or the C-terminal region of the molecule is interchanged [3]. Recent results on bovine pancreatic ribonuclease (RNase A) have proved that the capability to swap more than one region of the molecule may allow the formation of a variety of oligomeric organizations. Indeed, RNase A monomers, under well-defined experimental conditions, are induced to generate two types of dimers, based on the swap of either the N- or the C-terminus [4]. The protein is also able to form trimers and tetramers. Trimers are either generated by the swap of the C-terminus (cyclic trimers), or by the swap of both the N- and C-termini (linear trimers) [9].

Among the swapping proteins, bovine seminal ribonuclease (BS-RNase) [10], homologous to RNase A, presents unique features, since in its native form it is a homodimer, whose subunits are stably held together by two adjacent S–S bridges established between cysteines 31 and 32 of each monomer. An additional unique feature involves the fact that the native dimer is a mixture of two conformationally different isoforms, named MxM and M = M [11]. In the more abundant MxM isoform (about 70%), an interchange of the N-terminal α-helices (residues 1–15) between the two partner subunits occurs directed by the hinge peptide regions (residues 16–22) [12]. In the M = M isoform instead, 3D-domain swapping does not occur.

The two isoforms are in equilibrium and spontaneously interconvert into one another [11]. The conformational dimorphism of BS-RNase is a consequence of its unusual covalent dimeric structure, which does not allow monomerization upon unswapping. Only upon the selective reduction of the intersubunit disulfide bridges does the unswapped form freely monomerize, whereas the swapped form, due to the higher number of contacts at the intersubunit interfaces, is converted into non-covalent dimers [11]. Furthermore, the naturally dimeric BS-RNase can be induced to oligomerize under appropriate conditions. Tetramers, in which the four subunits are proposed to be enchained by multiple N-terminal swapping events, have been isolated and characterized [13].

BS-RNase isoforms present distinct functional properties. Only MxM is allosterically regulated, whereas M = M presents the typical Michaelis–Menten kinetics [11,14]. Interestingly, the swapped form is predominantly responsible for the well-established antitumor activity of BS-RNase [15,16]. Therefore,

*Corresponding author. Fax: (39)-081-674090.
E-mail address: mazzarella@chemistry.unina.it (L. Mazzarella).

Abbreviations: BS-RNase, bovine seminal ribonuclease; MxM, dimeric form of BS-RNase in which the chains swap their N-terminal helices; M = M, unswapped form of BS-RNase; M, monomeric BS-RNase; RNase A, bovine pancreatic ribonuclease; hRNase, human pancreatic ribonuclease; RI, ribonuclease inhibitor; rmsd, root mean square deviation

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the occurrence of 3D-domain swapping seems to have a physiological significance.

BS-RNase is thus a unique case of a metastable dimer, whose protomers naturally switch from a swapped to a non-swapped conformation. This phenomenon is evolutionarily puzzling and several hypotheses have been put forward [17–19].

The crystallographic structure of MxM in its native state and in complex with several substrate analogues has elucidated interesting aspects related to the protein function [12,20,21]. The monomeric form of BS-RNase has been recently characterized by nuclear magnetic resonance [22] and by X-ray crystallography [23]. In a previous study, crystals of the M = M isoform were obtained in two different conditions and space groups [24]. Here we present the crystal structure of the unswapped M = M form. This study represents the first example of the structural definition of a dimeric protein capable of 3D-domain swapping, isolated in the unswapped state.

2. Materials and methods

2.1. Notations

M = M consists of two subunits, each composed of three α-helices and a β-sheet core. The β-sheet core is V-shaped and is formed by residues 42–46, 61–63, 71–75, 82–87, 96–101, 105–111, and 116–124. Following the notation commonly used for 3D-domain swapping proteins, the region (residues 16–22) connecting the body of each subunit (residues 23–124) to its N-terminal helix (residues 3–13) is denoted the hinge peptide. Since active site residues belong to both the N-terminal helix and β-sheet regions, the interchange of N-terminal α-helices in the MxM form generates two composite active sites, whose residues are contributed by both subunits, whereas each active site of M = M is formed by residues belonging to the same chain. In the MxM form, structural units are defined as each composed of the body of one subunit and the N-terminal helix of the other [10].

2.2. Crystallization and data collection

The unswapped M = M form of BS-RNase was prepared as described by Piccoli et al. [11]. Particular care was devoted to protein purification and manipulation to avoid contamination by the MxM form. In particular, the sample was handled at a temperature of 4°C at pH 8.4, since in these conditions the rate of conversion to MxM is rather low [11]. Crystals from homogeneous M = M isoform were obtained in two different conditions and space groups [24]. For this study, we grew more ordered crystals, diffracting up to 2.2 Å, using the sitting drop method. Protein concentration was 11 mg/ml and the reservoir consisted of 28% (v/v) MPD, 50 mM Tris–HCl pH 8.4, 0.1 M ammonium phosphate. Crystals belonged to the $P2_1$ space group with unit cell parameters $a = 49.48$ Å, $b = 57.55$ Å, $c = 53.06$ Å, $\beta = 116.0°$. X-ray diffraction data to 2.2 Å were collected on a freshly grown crystal at 100 K using the synchrotron radiation at ELETTRA-Trieste. Data were processed with the HKL package [25]. Statistics of data processing are reported in Table 1.

Table 1
Crystallographic data

Space group	$P2_1$
Wavelength (Å)	1.0
Unit cell parameters (Å, °)	$a = 49.48$, $b = 57.55$, $c = 53.06$, $\beta = 116.0°$
Resolution range (Å)	15–2.2
Mosaicity (°)	0.7
Number of unique reflections	13 585
Completeness (%)	99.3 (99.9)
R_{merge} (%)	4.8 (8.6)
$\langle I \rangle / \langle \sigma(I) \rangle$	29.4 (15.1)
Redundancy	3.9
R-factor/*R*-free (%)	0.211/0.292
Average temperature factor (Å^2)	31.3

Values in parentheses refer to the highest resolution shell (2.24–2.20 Å).

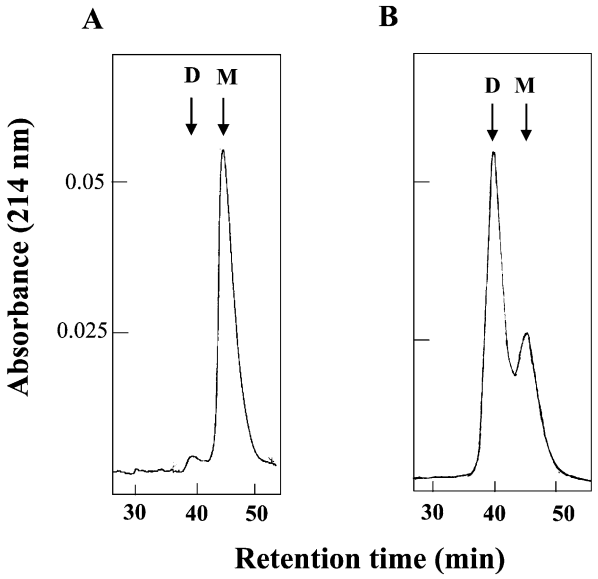

Fig. 1. Analysis of the isoform composition of M = M crystals. M = M from the dissolved crystals (a) and native BS-RNase (b) were analyzed for isoform content by FPLC gel filtration, after the selective cleavage of the intersubunit disulfides. D and M are the retention times of dimeric and monomeric species, respectively.

2.3. Structure solution and refinement

The structure was determined by molecular replacement with the AmoRe package [26] using the coordinates of the BS-RNase MxM structure (PDB code 1BSR) [12], deprived of the residues 1–22 for both subunits, as a search model. Structure refinement was carried out with the program XPLOR [27] using data in the 15–2.2 Å resolution range. The modeling of the two hinge fragments in the omit Fo−Fc density maps was performed manually using O [28]. Refinement cycles were then alternated by manual modeling of the structure. The model stereochemistry was checked using the programs PROCHECK [29] and WHATCHECK [30]. The final model, containing 1910 protein atoms and 107 water molecules, refined to an *R*-factor and an *R*-free of 0.211 and 0.292, respectively. Coordinates are being deposited in the Protein Data Bank (PDB, accession code 1R3M).

2.4. Chromatographic analyses

Crystals of the unswapped M = M form were dissolved after extensive washing in H_2O and analyzed for isoform composition as described by Piccoli et al. [11]. Briefly, after the addition of 1 M Tris-acetate at pH 8.4 to a final concentration of 0.1 M, the intersubunit disulfides were selectively reduced by dithiothreitol and subsequently alkylated with iodoacetamide. Products were then analyzed using a Hi-load Superdex 10/30 FPLC column (Pharmacia Biotech) equilibrated in 0.05 M ammonium acetate buffer at pH 5.0 and eluted at a flow rate of 0.3 ml/min. The same analysis was carried out on the mother liquor.

3. Results and discussion

3.1. Determination of the isoform content of crystals

The M = M isoform of BS-RNase has been crystallized from a pure M = M sample using novel crystallization conditions which disfavor 3D-domain swapping [11]. To verify the effects of the crystallization procedure on the protein composition of crystals, these were tested for the content of MxM and M = M isoforms [11]. The assay is based on the different molecular weight of the products obtained by the selective cleavage of the intersubunit disulfides of BS-RNase isoforms,

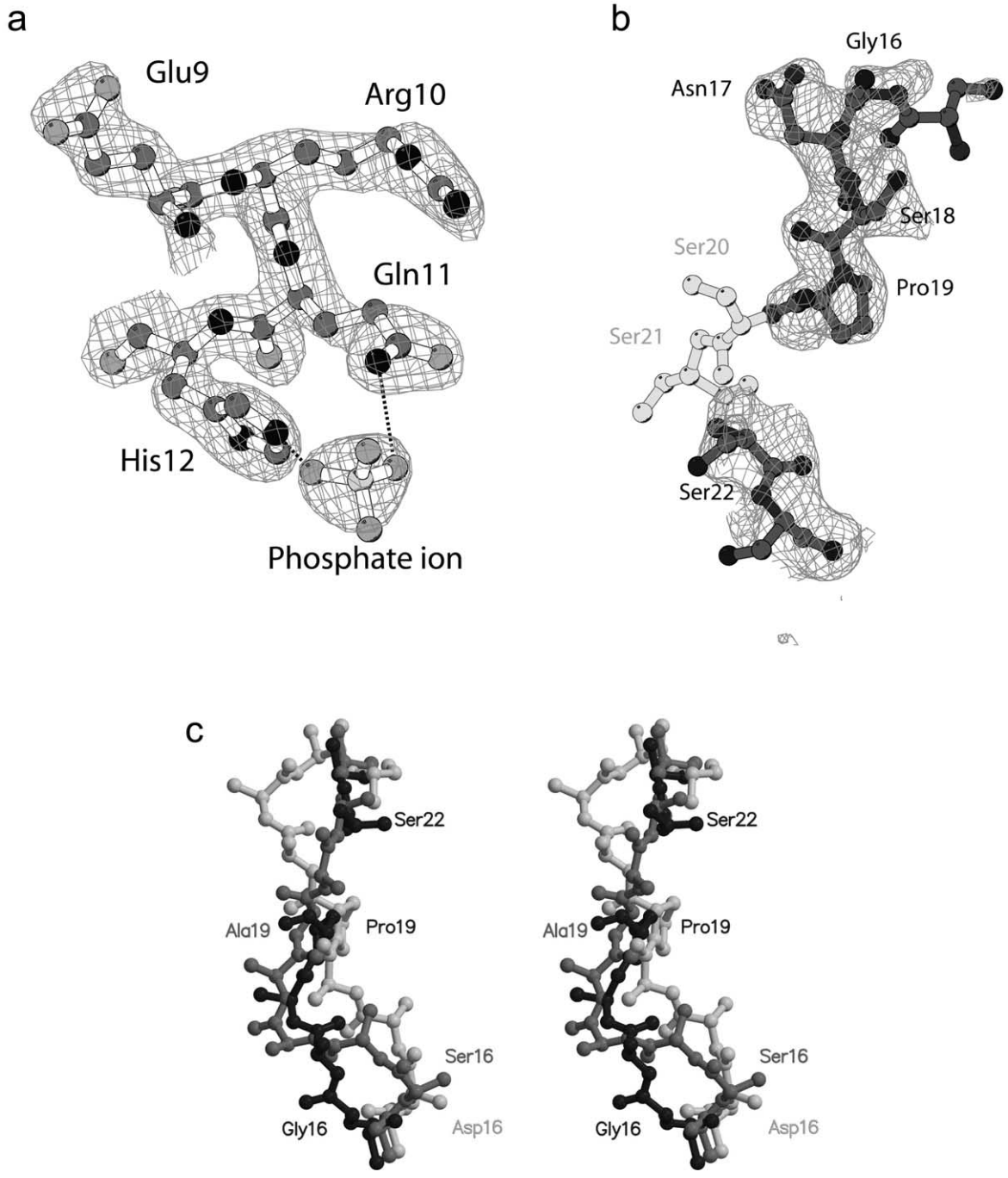

Fig. 2. a: Omit (Fo−Fc) map around a portion of the N-terminal helix including the catalytic His12. b: Omit (Fo−Fc) electron density around the hinge loop of one of the two subunits in the M = M crystal structure. Residues Ser20 and Ser21, which are fully disordered, are modeled (light gray) to show the hinge connectivity. c: Stereo diagram showing the hinge region after the superposition of a subunit of M = M (dark gray) with RNase A (medium gray) and hRNase (light gray).

i.e. monomers from M = M, and non-covalent dimers from MxM. Crystals were dissolved, and the protein content analyzed by gel filtration chromatography after the selective reduction of the intersubunit disulfides with dithiothreitol, followed by alkylation with iodoacetamide. As shown in Fig. 1a, a sole peak, whose elution volume corresponds to that of the monomeric species of BS-RNase, is eluted. For comparison, the chromatographic pattern obtained with native BS-RNase, a mixture of MxM and M = M forms, is also shown (Fig. 1b). Similar results were obtained from the analysis of the mother liquor (data not shown). These results indicate that the MxM isoform does not contaminate at any significant level the crystals of M = M.

3.2. Overall description of the protein

The quality of the electron density maps allowed a detailed description of nearly the whole M = M molecule, with the exclusion of the hinge peptide regions (residues 16–22) of each subunit, for which density maps are significantly less resolved (Fig. 2). The two subunits of the M = M isoform

Table 2
Interactions of the hinge loop regions

Hinge loop residue	M=M	RNase A	hRNase	MxM*
16	G16 N (I)-H48 O (I)	S16 N-D14 OD1		G16 N (I)-H48 O (II)
	G16 O (I)-R80 NH1(I)	S16 OG-D14 OD1		G16 O (I)-R80 NH1(II)
17	–	T17 OG1-D14 OD2	S17 N-D14 O	–
		T17 O-H48 ND1	S17 OG-D14 OD2	
18	S18 O (I)–R80 NE (I)	S18 O–S80 OG	–	S18 O (I)-S21 N (I)
19	–	–	–	–
20	NA	A20 N-Q101 OE1	S20 O-S23 OG	–
21	NA	–	–	S21 N (I)- S18 O (I)
22	S22 O (I)- Y25 N (I)	S22 O-Y25 N	S22 N-T99 O	S22 O (I)- Y25 N (I)
			S22 OG-T99 N	

	16	22
M=M	Asp-Ser-Gly-Asn-Ser-Pro-Ser-Ser-	**Ser-Ser-Asn-Tyr**[a]
MxM	Asp-Ser-Gly-Asn-	**Ser-Pro-Ser-Ser-Ser-Ser-Asn-Tyr**[a]
RNase A	**Asp-Ser-Ser-Thr**-Ser-Ala-Ala-Ser-	**Ser-Ser-Asn-Tyr**[a]
hRNase	**Asp-Ser-Asp-Ser**-Ser-Pro-	**Ser-Ser-Ser-Ser**-Thr-Tyr[a]

*Interactions common to all MxM structures available.
[a]Residues involved in β-turns are underlined.

share a strong structural similarity, as judged from their low main chain root mean square deviation (rmsd) of 0.27 Å. Each M = M subunit exhibits the typical fold of monomeric ribonucleases and is structurally similar to RNase A [31] (main chain rmsd close to 1.2 Å) and to human pancreatic ribonuclease (hRNase) [32] (rmsd close to 0.8 Å). Moreover, despite the occurrence of domain swapping, the structures of the two M = M subunits resemble those of MxM structural units (rmsd close to 0.5 Å), each composed of the body of one subunit and the N-terminal helix of the other (see Section 2 for definitions).

3.3. Active sites

A comparison of the active site architecture of M = M and MxM [12] isoforms indicated that no substantial differences occur, despite the composite nature of the active sites of the swapped form. In the present structure, a phosphate ion is bound to each M = M catalytic site and mimics the enzyme substrate (Fig. 2a). The strong similarity between the active sites of MxM and M = M provides structural bases for the previous biochemical data [11], indicating that the different catalytic behavior of MxM and M = M, the former of which is an allosteric enzyme, must be attributed solely to the cooperative interactions between MxM subunits, rather than to a different architecture of the active sites of the two isoforms. This observation is in accordance with the similar catalytic behavior of M = M to that of a hybrid BS-RNase swapped dimer with one of the catalytic sites inactivated [11].

3.4. Flexibility of the hinge regions

M = M isoform shows rather flexible hinge regions (residues 16–22). This was also described for the monomeric derivative of BS-RNase (M), obtained by the selective reduction of the intersubunit disulfides. However, whereas the hinge peptide of M was found to be fully disordered [23], the density maps of M = M hinge regions, although less resolved than for the rest of the structure (Fig. 2a), allowed modeling up to Ser18, and showed traces of density for Pro19 in one subunit (Fig. 2b). These findings are in line with a previous conformational analysis of the hinge peptide region of the monomeric derivative of BS-RNase, showing that the hinge peptide can adopt a wide range of different conformations, corresponding to comparable energy minima [33,34]. However, most of them were shown to be unsuited for the formation of non-swapped dimers, due to the formation of severe overlaps between residues 20 and 22 of one subunit and residues 22 and 20 of the other [34]. Therefore, the higher order observed for the M = M hinges, compared to that of M, is likely to derive from their restricted conformational freedom in the M = M dimer. Unlike M = M and M, the swapped form of BS-RNase, which is the most abundant, as well as most monomeric ribonucleases, present substantially structured hinge regions. Indeed, the hinge regions could be defined in all MxM structures known (Table 3), though their conformations significantly deviate from each other, depending on crystallization conditions and ligation state. Although this indicates a certain degree of flexibility, some interactions, mainly at the β-turn regions Ser18–Ser21, Ser21–Asn24 and Ser22–Tyr25, are rather conserved in the MxM structures studied so far. Similarly, hinge regions are structured in the naturally monomeric RNase A and hRNase.

To understand the structural bases of the lower flexibility of the hinge loops in these monomeric RNases and in MxM, the conformations adopted by the regions 16–18 of M = M have been compared to those of MxM, RNase A and hRNase. As a result, the conformations of the two M = M hinge regions, which are similar in the two subunits, differ substantially from those adopted in all the MxM structures defined so far (Table 3). Indeed, the lowest rmsd observed (from PDB code 1BSR) is close to 3 Å. It is worth noting that hinge loops are stabilized by three β-turns in MxM and by two β-turns in RNase A and hRNase (Table 2), whereas only one β-turn, encompassing residues 22 and 25, involves M = M hinges. As shown in Table 2, interactions of Gly16 of M = M with the neighboring residues differ from those of the correspond-

Table 3
Quaternary structure of BS-RNase isoforms

	M = M	MxM AS	MxM PEG	MxM	MxM	MxM
Ligation state	unligated	unligated	unligated	complexed with deoxycytidylyl-3′,5′-adenosine	complexed with uridylyl-3′,5′-guanosine	complexed with uridylyl-3′,5′-adenosine
PDB code	1R3M	1BSR	1R5D[a]	1R5C[a]	11BG	11BA
χ (°)	173.1	179.4	178.8	178.9	179.3	178.9
rmsd[b]	0.27	0.22	0.21	0.18	0.20	0.18

MxM AS and MxM PEG are crystallized from ammonium sulfate and from polyethylene glycol, respectively.
[a]A. Merlino, L. Vitagliano, F. Sica, A. Zagari and L. Mazzarella, submitted for publication.
[b]This refers to main chain rmsds after superposition of the two subunits.

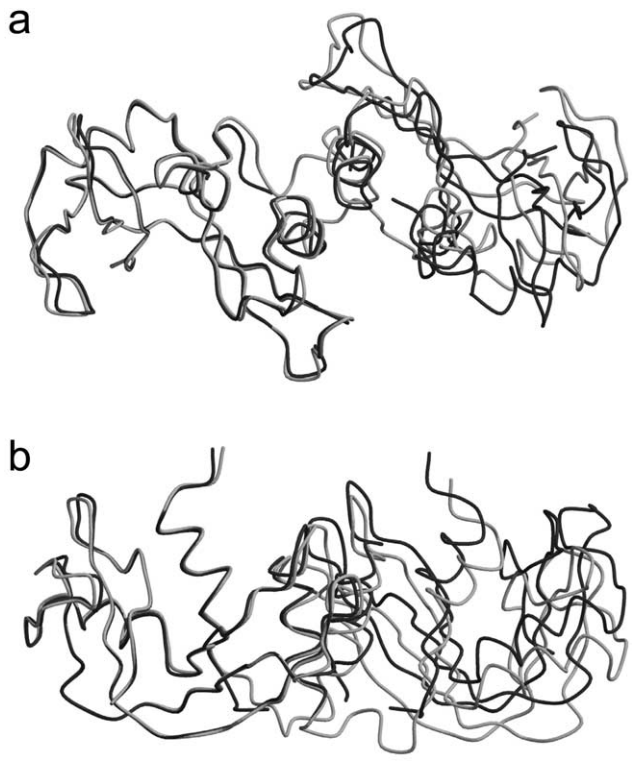

Fig. 3. Superposition of the C^{α} trace of one MxM subunit (PDB code 1BSR) (dark gray) with the corresponding M = M subunit (light gray). a: View along the approximate two-fold axis. b: A view of the C^{α} traces rotated by 90°.

ing residues in the two monomeric RNases. Consistently, the conformation of M = M hinge region deviates substantially from that of both RNase A and hRNase (Fig. 2c). We observed that such conformation would not be allowed to hinge fragments of RNase A and hRNase, since in M = M backbone Gly16 adopts ϕ, ψ angles which would not be accessible to other residues (average ϕ, ψ angles are 129.7° and −117.5°, respectively). On the other hand, with this conformation of the Gly16 backbone, the β-turn involving residues 14–17, which restrains the hinge conformation of monomeric ribonucleases, cannot be formed in M = M.

3.5. Analysis of the quaternary structure

The quaternary structure of M = M has been analyzed in comparison to those of the MxM crystal forms so far studied (Table 3). In all of these MxM structures, the swapping subunits are related by an approximately two-fold symmetry and, despite the different crystallization conditions and ligation states, the rotation angle relating the β-sheet core of the two subunits was found to be close to 180°. A lower symmetry instead characterizes M = M, with a rotation angle of 173° (Table 3 and Fig. 3a). The difference in the quaternary structures of M = M and MxM (PDB code 1BSR) is also shown by their stepwise superposition. After superposition of the β-core of one subunit, a further rotation of 12.5° is required to superpose the β-core of the second subunit (Fig. 3b).

The lower symmetry of the M = M quaternary structure indicates that, besides the two interchain disulfide bridges, which in both isoforms restrain the symmetry of the quaternary structure, further restraints are imposed by the swapping of the N-terminal helices in the MxM form. We verified that the different orientation of the subunits in M = M and MxM does not affect the geometry of the two disulfide bonds, which are located close to the rotation axis (Fig. 3a). Therefore, it is likely that, in the absence of the restraints due to 3D-swapping, crystal packing selects one of the possible conformations existing in solution.

Based on these results we propose that the flexibility of the unswapped structure may be mostly responsible for its observed non-cooperative catalytic behavior [11]. In previous reports [20] we showed that, upon binding of a substrate to MxM, a small variation of the protein tertiary structure occurs, namely a closure of the V-shaped structure of the bound subunits [20]. It is conceivable that in MxM the contraction of one subunit upon binding may require a similar contraction in the adjacent one, therefore limiting the access to its active site of a new substrate molecule. By contrast, due to the M = M flexibility, the conformational change of one of the M = M subunits upon binding is likely to be dispersed without reaching the adjacent subunit.

4. Conclusions

3D-domain swapping is suggested to be an important tool for regulation of the biological and enzymatic activities of BS-RNase, a unique case in protein polymorphism [14,15]. Swapped (MxM) and non-swapped (M = M) dimeric forms interconvert into one other in the absence of any ligands or effectors and only the MxM isoform is responsible for the allosteric behavior and the antitumor activity of BS-RNase.

The crystal structure of M = M, here reported at 2.2 Å resolution, shows that the overall protein shape resembles that of MxM [12]. This strong structural similarity of BS-RNase isoforms explains the observation that both isoforms are able to escape the RNase inhibitor (RI) [35], a very effective inhibitor of monomeric pancreatic-like RNases [36]. Therefore, the inability of M = M to act as an effective antitumor agent [15] has to be related to its higher propensity, compared to MxM [37,38], to monomerize in the cytosolic compartment of mammalian cells, where the reducing environment may lead to the breakage of intersubunit disulfide bonds [39]. Indeed, monomerization renders the protein unable to escape RI.

The comparison of the active site regions of M = M and MxM showed that the swapping event does not have any direct structural implications on the active site architecture. These findings support previous biochemical data indicating that the different catalytic behavior of M = M and MxM is to be attributed solely to cooperativity effects [11].

A rather pronounced flexibility of the hinge regions, which modulates 3D-domain swapping, was observed in M = M. It is interesting to note that such pronounced flexibility of the hinges, also found in the monomeric derivative of BS-RNase [23], is not a feature of the swapped form. This observation highlights an active role of the hinge peptides as possible structural determinants responsible for the communication between the two composite active sites in the MxM form. Along this line, well-defined hinge peptides are not physiologically required in M = M, which does not exhibit allosteric properties.

Intriguing is the finding that, despite the fact that M = M and MxM present identical sequences, the quaternary structure of M = M is characterized by a less symmetric organiza-

tion of its subunits. This result, along with the constant tight two-fold symmetry observed in MxM (Table 3), may be attributed to a higher flexibility of M = M, due to the absence of those restraints induced by the swapping of the N-terminal helices.

We may surmise that, due to this flexibility of the unswapped form, subtle conformational alterations, induced in one of the subunits upon substrate binding, are likely to be easily dissipated without reaching the adjacent subunit. Thus, our data offer a molecular basis to explain the non-cooperative behavior of the unswapped form of BS-RNase and complete the structural definition of the conformational dimorphism of the protein.

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