BS-RNase tetramers: an example of domain-swapped oligomers

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Abstract In the ribonuclease superfamily, dimericity is a unique feature of bovine seminal RNase (BS-RNase). In about two-thirds of native BS-RNase molecules, the two subunits interchange their N-terminal tails, thus generating domain-swapped dimers (MxM), which mostly responsible for enzyme biological activities and allostericity. Higher molecular weight BS-RNase oligomers can also be prepared [Libonati, M. (1969) Ital. J. Biochem. 18, 407–417.]. This paper reports on BS-RNase tetrameric derivatives which were isolated and enzymatically characterized. The data collected and the analysis of the crystal packing of MxM dimers suggested a structural model for tetramer assembly, in which the four subunits are enchained by multiple domain-swapping events.

Key words: Seminal ribonuclease; Domain swapping; Oligomeric structure

1. Introduction

Bovine seminal ribonuclease (BS-RNase), a homodimeric ribonuclease [1] endowed with unusual biological action [2], is the only dimeric RNase isolated so far with two interchain disulfides linking the two subunits. It can be isolated from bull seminal plasma or bull seminal vesicles [3], the organ where it is produced. BS-RNase is an allosteric enzyme, regulated by the cyclic nucleotide substrate analogues of the second rate-limiting step of the ribonucleolytic reaction, through mixed-type cooperative effects [4].

The enzyme exists as two distinct conformational isomers [5] (see Fig. 1a): a major form (MxM), well characterized by X-ray crystallography at 1.9 Å resolution [6], in which the two subunits interchange their N-terminal α-helices, as proposed by Fruchter and Crestfield [7] for artificially dimerized RNase A, and a minor form (M=M), in which the interchange does not occur. In native BS-RNase, the two forms, MxM and M=M, are in a constant 2:1 concentration ratio [5].

The two forms can be isolated, after selective reduction of the intersubunit disulfides, by means of gel filtration and air reoxidation [5]. The products of the selective reduction of MxM and M=M differ in molecular mass: the reduction product of MxM is a non-covalent dimer, in which the quaternary structure is maintained by non-covalent interactions between each N-terminal tail and the body of the other chain, whereas the reduction product of M=M is a monomer.

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Abbreviations: BS-RNase, bovine seminal RNase; RNase A, bovine pancreatic RNase; cyd-2′:3′-P, cytidine-2′:3′-cyclic phosphate; DTT, dithiothreitol; DFDNB, 1,5-difluoro-2,4-dinitrobenzene

An important consequence of the structural arrangement of the MxM form is the composite nature of its active sites [5,8], where the catalytically essential residues belong to different subunits. Kinetic studies on the isolated forms of BS-RNase [5] have shown that only the MxM isomer is endowed with allosteric properties. Furthermore, of the two forms, the MxM isomer was found to be responsible for the major part of the antitumor activity of native BS-RNase [9]. Therefore, the interchange of the N-terminal segments represents the molecular basis for both the allosterism and antitumor action of BS-RNase.

The domain-swapping phenomenon [10–12], which has also recently been described for other oligomeric proteins ([12], and references therein), allows a significant increase of the subunit interfaces, without altering the whole architecture of the oligomer (see Fig. 1a).

Tetramers and higher molecular weight aggregates of BS-RNase, generated by lyophilizing the protein from concentrated acetic acid, were first described by Libonati [13]. In the present paper, we describe the isolation and characterization of BS-RNase tetramers; kinetic and structural analyses suggest an intriguing structural arrangement of BS-RNase tetramers. On the basis of the results obtained, a model is proposed, in which the tetrameric structure is maintained by the N-terminal α-helices swapping among the four subunits.

2. Materials and methods

2.1. Tetramer purification

BS-RNase higher oligomers were generated by protein lyophilization from 40% acetic acid [13]. The products were separated by gel-filtration chromatography on a Sephadex G-100 column (1.6×90 cm), equilibrated in 0.2 M potassium phosphate buffer, pH 6.5. Tetramer-containing fractions were pooled and applied to a Hi-load Superdex 10/30 FPLC column (Pharmacia), equilibrated in phosphate buffer as above, at a flow rate of 0.3 ml/min.

2.2. Molecular mass determination of tetrameric BS-RNase

Tetrameric BS-RNase (50 μg) was analyzed by: (a) HPLC gel-filtration chromatography (Perkin Elmer), on a Protein Pak 125 column (Waters), equilibrated in 0.2 M phosphate buffer, pH 6.5, at a flow rate of 0.6 ml/min; (b) SDS-polyacrylamide (12%) gel electrophoresis, performed as described [14]. In this case, the cross-linked tetramer (see below) was used.

Bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), BS-RNase (27.4 kDa) and RNase A (13.7 kDa) were employed as standards for the molecular mass calibration curves.

2.3. Preparation of cross-linked tetramers

A cross-linked stable derivative of tetrameric BS-RNase was prepared with 1,5-difluoro-2,4-dinitrobenzene (DFDNB), following the procedure described in [15], with minor modifications. Briefly, tetrameric BS-RNase (1.2 mg) was dissolved in 6 ml of 50 mM sodium borate buffer, pH 8.5. DFDNB (Sigma, 0.18 μmol in 0.48 ml) was added to the protein solution, at a flow rate of 0.6 ml/h. The reaction mixture was stirred for a further 20 h in the dark. To stop the reaction, the pH value was lowered to 3 with acetic acid.

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2.4. Kinetic analyses

The cyclic nucleotide cytidine-2′:3′-P (cyd-2′:3′-P) was purchased from Sigma Chemical Co. (St. Louis, MO). The enzymatic assays were performed in 0.1 M Tris-HCl, pH 7.3, at 25°C, as described [4]. The enzyme concentration was 0.4 μM. The assays with yeast RNA were performed as in [16].

2.5. Determination of N-terminal exchanging structures

Tetrameric BS-RNase (50 μg in 100 μl) was selectively reduced in 0.2 M potassium phosphate buffer, pH 8.0, adjusted to pH 8.4 with diluted NaOH, in the presence of a 5×molar excess of DTT (Sigma) with respect to the intersubunit disulfide bridges, as described [17]. The exposed sulphydryls were then reacted for 45 min in the dark with iodoacetamide, in a 2×molar excess with respect to DTT. The products were analyzed using a Hi-load Superdex 10/30 FPLC column (Pharmacia), equilibrated as described above.

3. Results and discussion

Higher oligomers of BS-RNase were generated by lyophilization of native dimeric BS-RNase from 40% acetic acid [13]. The products were separated by gel-filtration chromatography on a Sephadex G-100 column. The chromatographic pattern (Fig. 2) revealed the presence of multiple BS-RNase aggregates. The fractions indicated in the figure, corresponding to the elution volume of the tetrameric species, were purified on a Hi-load Superdex 10/30 FPLC column. A single peak was obtained, as shown in the inset of Fig. 2.

The presence of the phosphate in the chromatographic buffer is essential for stabilizing the tetrameric structure, since no aggregates were obtained when the lyophilized products were gel-filtered in 0.05 M ammonium acetate buffer at pH 5 (data not shown). Furthermore, BS-RNase tetramers have been found to be fully stable for at least 1 day if stored at 4°C in phosphate buffer; under these conditions, only 13% of tetramers dissociate into dimers after 2 weeks. All the subsequent analyses were carried out immediately after preparation of the tetramers.

The apparent molecular mass of tetrameric BS-RNase was calculated by HPLC gel-filtration chromatography (see Section 2). From the calibration curve, shown in Fig. 3a, the molecular mass was estimated to be 53.7 kDa, a value comparable to the expected value of a tetrameric derivative of BS-RNase (54.8 kDa). Furthermore, to determine the molecular mass of tetrameric BS-RNase also under denaturing conditions, i.e. by SDS-PAGE, covalent stabilization of the tetrameric structure was necessary. For this purpose, the tetramer was reacted in the presence of the cross-linking agent 1,5-difluoro-2,4-dinitrobenzene (see Section 2). The cross-linked products were analyzed by SDS-gel electrophoresis, as shown in Fig. 3b: in the absence of a reducing agent (lane 2), two bands were present, corresponding to BS-RNase tetramer and dimer, respectively, whereas in the presence of the reducing agent (lane 1) four bands were detected, corresponding to tetra-, tri-, di- and monomeric BS-RNase derivatives, as expected for a cross-linked product of a tetrameric species. The tetramer molecular mass was 57.5 kDa, as estimated from the calibration curve shown in Fig. 3a.

The enzymatic activity of BS-RNase tetramer was determined on yeast RNA, as well as on the cyclic nucleotide cyd-2′:3′-P, as substrates for the first and second steps of the ribonucleolytic reaction, respectively. The results are summarized in Table 1. Comparison between the specific activities of tetrameric BS-RNase and native dimeric BS-RNase shows that the tetramer is 10-fold less active on RNA with respect to the native enzyme, but fully active on the cyclic nucleotide. Similar results with RNA have also been described in [13].

The saturation curve of tetrameric BS-RNase with cyd-2′:3′-P is shown in Fig. 4. A non-hyperbolic curve, similar to that described for native BS-RNase [4], was obtained, revealing a mixed-type cooperativity [18] among the tetramer sites. The integrity of the tetrameric structure under the assay conditions [4] was confirmed by FPLC gel-filtration analysis of the tetramer, after 5 min incubation at 25°C in the assay buffer, containing 3 mM cyd-2′:3′-P (data not shown).

As the cooperative behaviour of native BS-RNase is associated only with the domain-swapped MxM dimer [5], the existence of interchanged N-terminal segments between the tetramer subunits was hypothesized. This hypothesis was also based on the analysis of the crystal packing of the

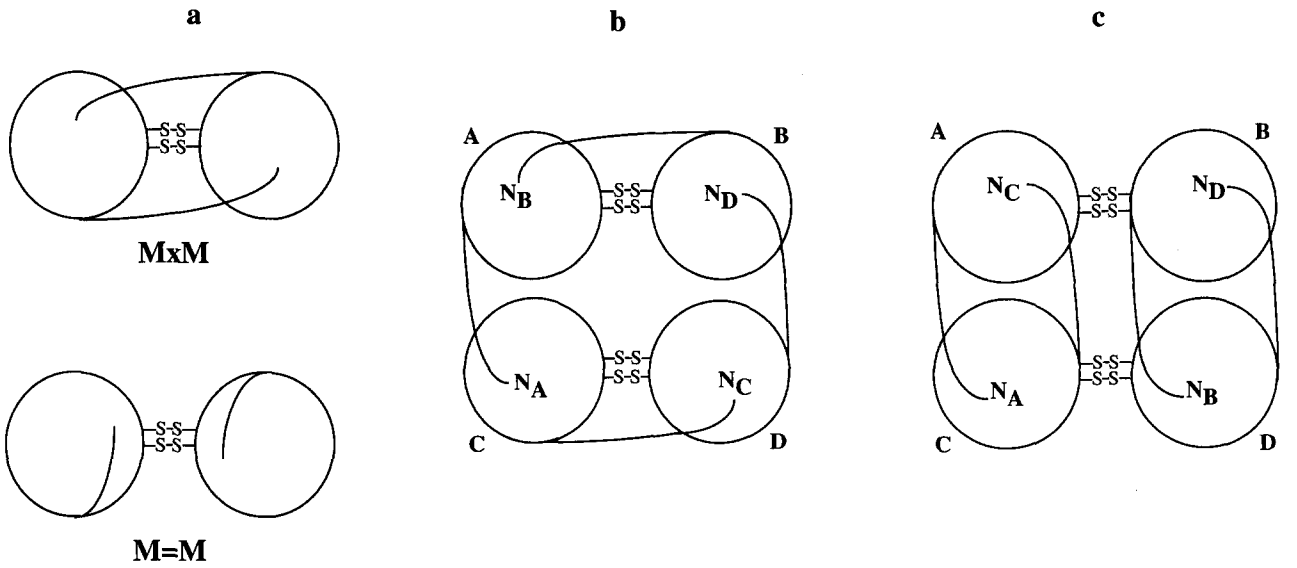

Fig. 1. (a) The two quaternary structures of BS-RNase; (b) the cyclic swapped tetramer structure; (c) the double swapped tetramer structure. N_A, N_B, N_C, N_D, N-terminal ends of subunits A–D, respectively.

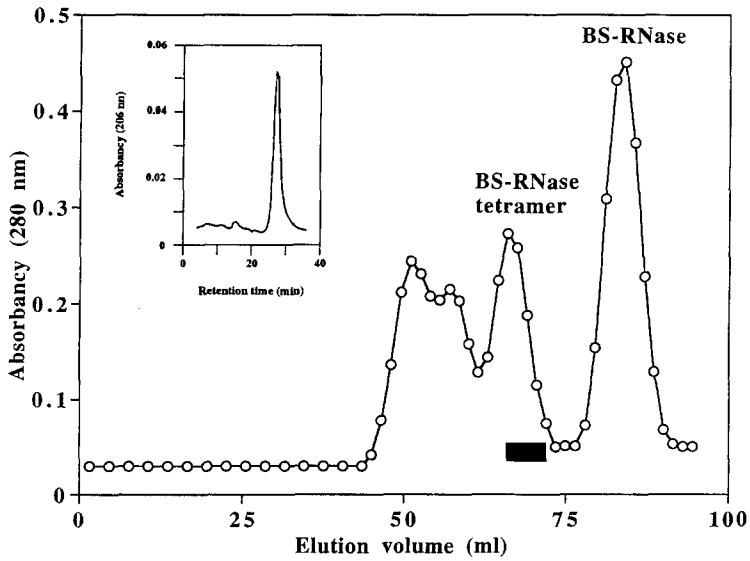

Fig. 2. Gel filtration on a Sephadex G-100 column of BS-RNase lyophilized from 40% acetic acid. The fractions indicated were pooled and repeatedly purified by FPLC. The homogeneous sample, analyzed by FPLC, is shown in the inset.

MxM dimer [6], that suggests a convincing structural model for the tetrameric aggregates of BS-RNase. In the crystal, which has space group symmetry $P22_12_1$, the dimer is oriented with its molecular (non-crystallographic) 2-fold axis nearly parallel to the *c* axis and normal to the crystallographic 2-fold axis along the *a* axis; therefore, two dimers are packed back to back with an approximate 222 symmetry. A view of this tetrameric association in the crystal is shown in Fig. 5A, projected along the crystallographic 2-fold axis (A–D denote the four subunits). Swapping of the N-terminal occurs between A and B (upper dimer) and between C and D (lower dimer). The arrangement of the two dimers forms an extensive contact surface, which includes residues of the β sheet structure and the region of the hinge peptide (residues 16–22) of each subunit. However, as native BS-RNase is not aggregated in solution, these interactions are not sufficient to stabilize the tetramer structure in solution.

An interesting feature of the dimer-dimer interactions in the crystal lies in the fact that the distance covered by the hinge peptide connecting the body (residues 23–124) of subunit A and its N-terminal segment (residues 1–15), folded on subunit B of the swapping dimer, is approximately equal to that between residue 22 of subunit A and residue 16 of the N-terminal segment folded on subunit C. By virtue of the crystallographic 2-fold axis, this is also true for the connection between the body of D with respect to the N-terminal peptide folded on B. Therefore, within the framework of this tetrameric structure, we can envision a conformational change of the hinge peptide which allows the switch of the N-terminal fragment of A from B to C and that of the N-terminal fragment of D from C to B. The resulting tetramer would be characterized by cyclic swapping of the N-terminal fragments according to the scheme A→C, C→D, D→B, B→A, as depicted in Fig. 1b. A tentative model of the A and D hinge peptides, based on the MxM crystallographic coordinates, indicates that this scheme can be achieved by repositioning only four residues and does not produce disallowed contacts (Fig. 5B). It should be stressed, however, that a meaningful modeling of the structure would require an exhaustive conformational analysis of the entire hinge peptide [19] and a finer adjustment of the relative position of the two dimers.

The cyclic swapping of the N-terminals enchains the four subunits together, providing an extensive interacting surface which stabilizes the tetrameric structure even in the absence of the interchain disulfides, as occurs for the MxM dimers, after the selective reduction of the intersubunit disulfides [5].

With reference to Fig. 5A, one may note that a further swapping, similar to that described above, can occur between the symmetry-related pairs of the N-terminal segments belonging to subunits B and C; the resulting tetramer, sketched in Fig. 1c, is characterized by a double interchange of the N-terminals between the pairs A/C and B/D, respectively, and does not present cross-swapping between the pairs of subunits linked by the interchain disulfides.

To test this hypothesis, i.e. the presence of exchanging structures in the tetrameric molecules, the intersubunit disul-

Table 1
Enzymatic activity of tetrameric BS-RNase

	RNA		Cyd-2′:3′-P	
	U/mg[a]	%	*v*/[E][b]	%
BS-RNase	27.9 ± 0.1	100	1.53 ± 0.01	100
Tetrameric BS-RNase	2.7 ± 0.1	9.8	1.64 ± 0.01	107

[a]Calculated from Kunitz assay [16].
[b]Expressed as μmol/l of substrate transformed in 1 min by 1 μg of enzyme. The assays were performed in 0.1 M Tris-HCl, pH 7.3 at a 0.35 mM substrate concentration.

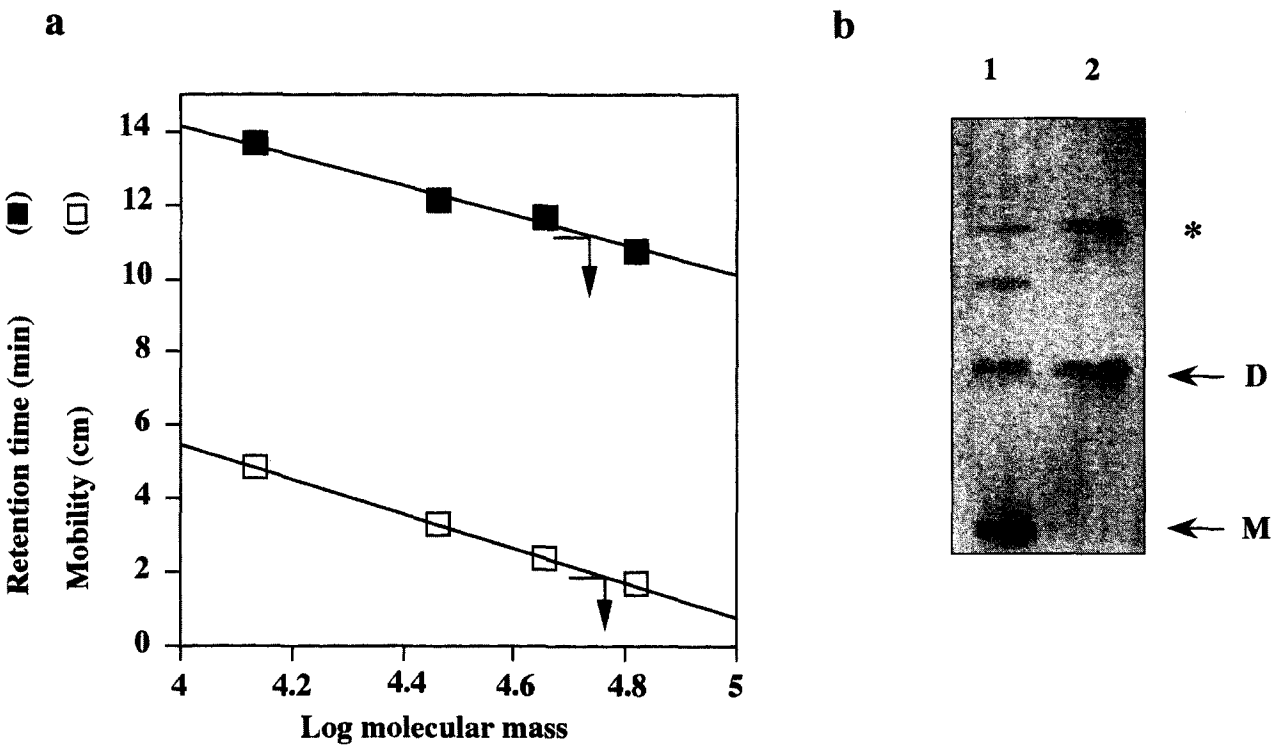

Fig. 3. (a) Molecular mass determination of BS-RNase tetramer: calibration curves from SDS-PAGE (□) and gel-filtration chromatography (■). Arrows indicate the position of tetrameric BS-RNase. (b) SDS-polyacrylamide (12%) gel electrophoresis of cross-linked BS-RNase tetramers, under reducing (lane 1) and non-reducing (lane 2) conditions. Arrows indicate the positions of standard dimeric (D) and monomeric (M) BS-RNase. The asterisk denotes the band corresponding to a tetrameric molecular mass.

fides were selectively reduced with DTT, as described in Section 2. After reduction, the exposed sulphydryls were alkylated with iodoacetamide to avoid air reoxidation. A sample aliquot was analyzed by SDS-gel electrophoresis: the presence of a single monomeric species (data not shown) confirmed the complete reduction of the intersubunit disulfides. The reduction products were then analyzed by FPLC gel-filtration chromatography (Fig. 6a). More than 75% of the protein was eluted earlier than native BS-RNase. Two main species were present with an elution volume corresponding to those of tetrameric and trimeric aggregates of BS-RNase, respectively.

These results indicate that, after the cleavage of the intersubunit disulfides of the two dimeric units that compose the tetramer, non-covalent interactions are still responsible for the structural arrangement of higher BS-RNase oligomers. The existence of non-covalently linked tetrameric species (see Fig. 6a) indicated that the tetramer quaternary structure is that proposed in Figs. 1b and 5B, where the four subunits are enchained by cyclic swapping of the N-terminal ends. Upon reduction of the interchain disulfides, partial unfolding of the N-terminal segments may give rise to trimeric, dimeric, and monomeric species, as shown in Fig. 6a. On the other hand, dimers, which represent less than 15% of the total reduction products, could also be generated upon the selective reduction of the double swapped tetramer structure proposed in Fig. 1c.

Thus, on the basis of the results of Figs. 4 and 6a, we conclude that the swapping of the N-terminal tails among the subunits of the tetramer represents the molecular basis for the tetrameric BS-RNase architecture. Moreover, considering the two quaternary structures proposed in Fig. 1b,c, the data available indicate that the majority of the tetrameric molecules are arranged in the cyclic swapped quaternary structure represented in Fig. 1b. In a minority of tetrameric molecules, a second swapping event between subunits belonging to different dimers may occur, thus generating the quaternary structure represented in Fig. 1c.

As previously demonstrated [5,8], N-terminal swapping events between BS-RNase subunits generate composite active sites, in which the two catalytic histidines 12 and 119 belong

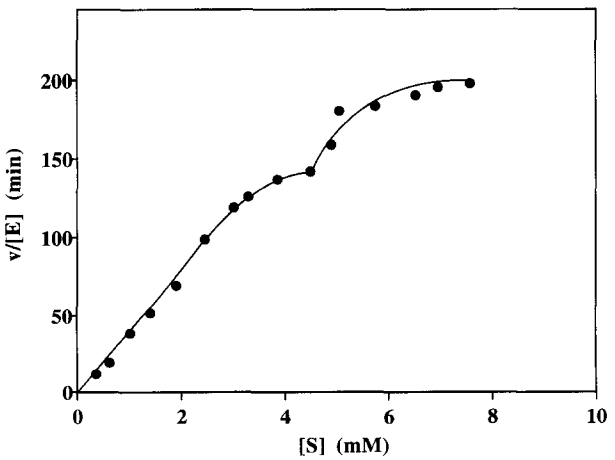

Fig. 4. Saturation curve of tetrameric BS-RNase with cyd-2′:3′-P. The spectrophotometric assays were performed at room temperature, in 0.1 M Tris-HCl, pH 7.3, at 0.4 μM final enzyme concentration.

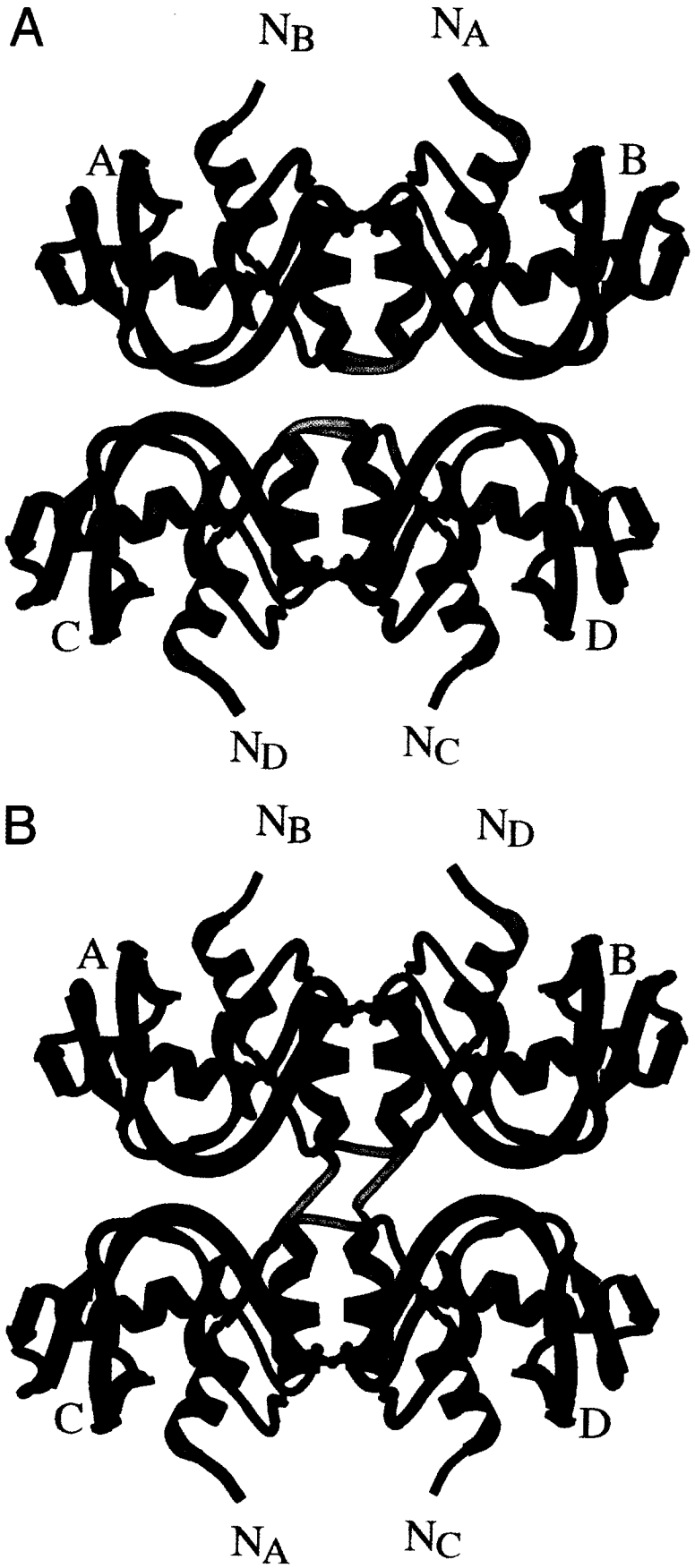

Fig. 5. (A) Crystal packing of MxM dimer, projected along the crystallographic 2-fold axis; (B) model proposed for the cyclic swapped tetramer structure. N_A, N_B, N_C, N_D, N-terminal ends of subunits A–D, respectively.

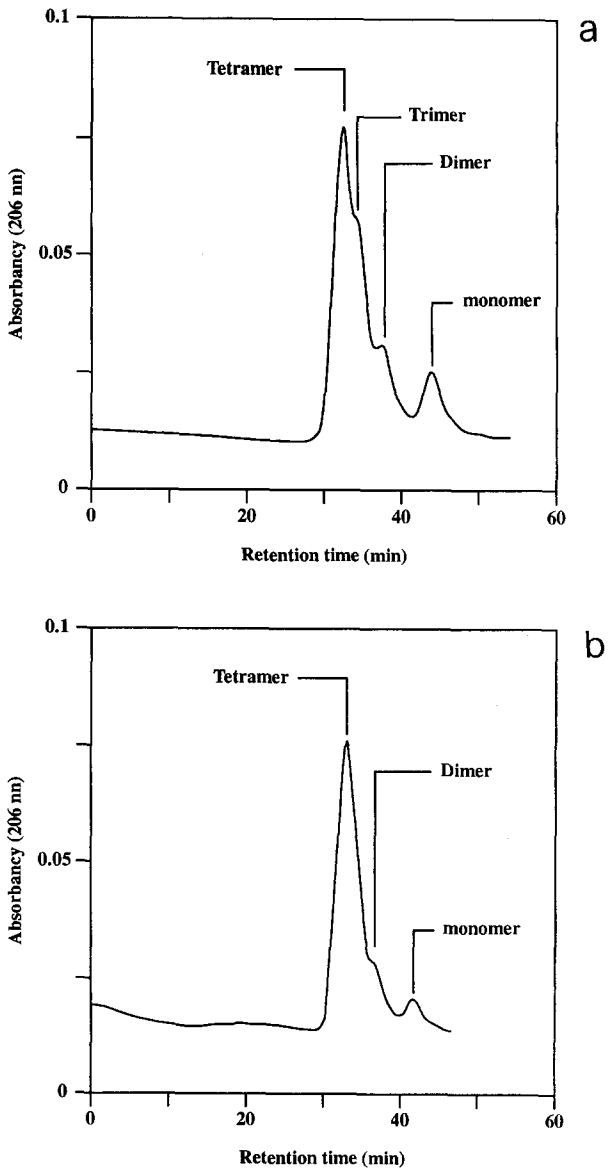

Fig. 6. FPLC gel-filtration analysis of the selectively reduced tetramer (see Section 2), in the absence (a) or presence (b) of 20 mM cyd-2′:3′-P.

to different chains. In both structures proposed for BS-RNase tetramers (Fig. 1b,c) all of the tetramer active sites are composite. In this case one would expect that the binding of a substrate analogue to the tetramer active sites would stabilize the non-covalent interactions in the oligomeric structure, by holding together structural domains belonging to different subunits. This phenomenon has recently been described for BS-RNase [20].

In order to analyze the effect of the substrate on the tetramer stability, the experiment of Fig. 6a was repeated in the presence of 20 mM cyd-2′:3′-P. The results are shown in Fig. 6b. In the presence of the substrate, even after cleavage of the intersubunit disulfides, the great majority of the protein maintains its tetrameric structure. In Fig. 6b, both the absence of trimers and the significantly lower amount of dimers and monomers with respect to Fig. 6a indicate that the binding of the substrate to the composite active sites stabilizes the quaternary structure of the tetramer.

In conclusion, the data collected indicate that multiple domain swapping events, induced by lyophilization at low pH and stabilized by active site binding molecules, represent the structural basis for BS-RNase tetrameric architecture.

The two models presented in Fig. 1 arise from the unique feature of BS-RNase of two intersubunit disulfides, which impose a 2-fold symmetry axis on the molecule. As the N-terminus of one chain is absorbed on the chain of a different dimer, further swapping is facilitated by the proximity of the second subunit in each dimer, and, therefore, closed domain-swapped tetramers are favored. However, aggregates higher than tetramers are also present in the protein lyophilized from acetic acid (see Fig. 2), suggesting that linear domain-swapped oligomers [12] can also be formed.

The differences between the two tetramers in Fig. 1 are strictly confined to the region of the hinge peptides, and the relative population of the two tetramers would be mainly determined by the difference in energy of the hinge peptide conformations required for the swapping of the N-termini within or across the dimers. This consideration also applies to the native enzyme for the swapping that gives rise to the equilibrium between the two isomers MxM and M=M [5,19].

It is conceivable that the high concentration of BS-RNase in seminal plasma may favor aggregated forms of the protein; whatever their biological relevance might be, we can conclude that the domain-swapping process in BS-RNase allows this protein to reach at least two levels (dimers and tetramers) of well-defined quaternary structural organization of increasingly higher complexity, which may be of general interest in the evolution of oligomeric proteins.

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