Dimeric seminal RNase presents the singular case of a dimer with access at equilibrium to two conformations: in one which the subunits exchange, or swap, their NH$_2$-terminal arms; the other with no exchange. Thus a continuous unfolding/refolding of structural elements into two alternative conformations takes place in the native protein at equilibrium. The phenomenon was investigated by kinetic and mass spectrometric analyses of the effects of trypsin on the native protein, on its isolated quaternary forms, as well as on a monomeric derivative of the protein and on homologous dimeric RNase A. The kinetics of tryptic action on the protein forms and on the protein derivatives, as well as the location of the tryptic cleavage sites, and their chronological sequence, led to the identification of relevant interconversion intermediates, to the description of a model for the interconversion process, and to a hypothesis for the unique phenomenon of the dual quaternary conformation of seminal RNase.

According to Anfinsen's thermodynamic hypothesis (1), a polypeptide chain spontaneously folds into its most stable conformation. This may be true only when conformations kinetically accessible "under normal conditions" are considered (2). Thus it may not be surprising to register cases of proteins that upon an influential change of conditions have access to two conformations: one monomeric, the other oligomeric (3–7).

Protein chains that spontaneously fold and associate into two distinct quaternary structures in equilibrium are a different story. This is the case of bovine seminal RNase (BS-RNase$^1$; for a review, see Ref. 8), a dimeric protein from the pancreatic-type family (9), an RNase also endowed with special, i.e. noncatalytic, biological actions. For this protein two quaternary structures have been described. In one structure (termed MxM), determined by x-ray crystallography (10), the two subunits interchange their NH$_2$-terminal $\alpha$-helices (see Fig. 1A). An alternative structure, in which there is no interchange (termed M=M, see Fig. 1E) has been determined by biochemical analyses (11) and recently observed by x-ray crystallography (12).

It has been found (11) that when the two quaternary forms of BS-RNase are isolated, they slowly convert into each other under pseudophysiological conditions until the equilibrium ratio of 2:1 is reached between the MxM and M=M forms. Thus, native BS-RNase as isolated, or upon refolding after denaturation and reduction of all disulfides (11), may be better described as an equilibrium mixture composed of MxM and M=M forms plus all of the transient intermediates in the interconversion process. This interconversion may also be viewed as a steady-state unfolding/refolding of the protein regions comprising the NH$_2$-terminal arms and the hinge peptides connecting them to the main bodies of the subunits.

Our aim was to investigate this unusual phenomenon not only for the intrinsic interest in its structural aspects but also for its possible relevance to the special biological actions of the enzyme, in particular to its antitumor action, given the correlation suggested between the antitumor activity of seminal RNase and its content in the swapped form (13–15).

Recently, trypsin was tested on the isolated BS-RNase forms and found to be capable of discriminating between MxM and M=M (16). Different limit digest products (see Fig. 1, panels D and H) were obtained from the two forms after extensive digestion, with both products enzymatically active and stable.

Here we report on the use of trypsin as a conformational probe to investigate the kinetics and the molecular basis of the MxM $\leftrightarrow$ M=M interconversion process. The results of this investigation led to the identification of relevant intermediates in the interconversion and to the description of a model for the interconversion process. This in turn shed a first light on the unusual case of a protein in which two quaternary conformations coexist.

**EXPERIMENTAL PROCEDURES**

Limited Proteolysis of BS-RNase and Its Quaternary Forms—BS-RNase was isolated from bovine seminal vesicles as described (17); the preparations of the isolated quaternary forms MxM and M=M (11) and of the M-Cm monomeric derivative (18) of seminal RNase and of dimeric RNase A (19) have been described previously. The S protein fragment of RNase A was a Sigma product.

For kinetic analyses, unless otherwise stated, the proteins were incubated with 0.2 $\mu$m trypsin (Sigma) in 0.1 M Tris acetate, pH 8.4, at 37 °C, with an enzyme to substrate ratio of 1:50 (w/w). Aliquots of 10 $\mu$g were withdrawn, and the reaction was stopped by adding soybean trypsin inhibitor in a 10-fold excess (w/w) with respect to the enzyme. Products were analyzed by SDS-polyacrylamide (15%) gel electrophoresis (20) or by RP-HPLC (reverse phase high performance liquid chromatography) as described (16).

For mass spectrometric analyses, the proteolytic experiments were carried out by treating the isolated dimeric forms (38 $\mu$m) of BS-RNase with 0.4 and 0.9 $\mu$m trypsin, for M=M and MxM, respectively, at 37 °C.

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$^\ddagger$The abbreviations used are: BS-RNase, bovine seminal RNase; MxM, dimeric form of BS-RNase with exchange of end terminal ends between subunits; M=M, dimeric form of BS-RNase without exchange; M-Cm, monomeric derivative of BS-RNase; I, intermediate; RP-HPLC, reverse phase high performance liquid chromatography; ESMS, electrospray mass spectrometry; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry.

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Trypsin Sheds Light on the Singular Case of Seminal RNase, a Dimer with Two Quaternary Conformations*

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**RESULTS AND DISCUSSION**

**Limited Tryptic Proteolysis of BS-RNase and of Its Isolated Quaternary Forms**—Native, as isolated BS-RNase (i.e. the equilibrium mixture of its MxM and M–M forms) was treated at a concentration of 66 μM at 37 °C with 1.5 μM trypsin in 0.1 M Tris acetate at pH 8.4. The reaction was stopped by the addition of soybean trypsin inhibitor. Analysis by SDS-gel electrophoresis of the tryp tic products, illustrated in Fig. 2A, shows that the effect of trypsin is limited, as discrete degradation fragments are formed. Moreover, only a small fraction of BS-RNase is cleaved by trypsin under the conditions employed. A fragment of about 20 kDa is detected first, which decreases at longer intervals of incubation time; then a smaller (17-kDa) product forms and accumulates. The same trypsin treatment on the isolated MxM and M–M forms produced identical patterns of products, but at faster rates, especially when the M–M

**Interconversion of Dimeric Forms of BS-RNase**—Homogeneous MxM and M–M preparations, obtained as described (11), were incubated at 37 °C at a concentration of 15 μM in 0.1 M Tris acetate, pH 8.4, in the absence or in the presence of trypsin (0.3 μM). At suitable time intervals aliquots (30 μg) were tested for the MxM and M–M content as described (11). Briefly, after the selective reduction of the two intersubunit disulfides, noncovalent dimers and monomers were generated from the MxM and M–M form, respectively. The relative percentage of the dimeric and monomeric species was calculated after gel filtration on a Hi-load Superdex 10/30 fast protein liquid chromatography column (Amersham Pharmacia Biotech), equilibrated in 50 mM ammonium acetate buffer, pH 5.0, containing 0.3 M sodium chloride, at a flow rate of 0.3 ml min⁻¹. An aliquot (5 μg) of the reduced samples was analyzed by SDS-polyacrylamide (15%) gel electrophoresis (20) to verify that disulfide reduction was complete. Because the monomeric derivative (13.7 kDa) coeluted in the gel filtration pattern with the 17-kDa tryp tic product, for an accurate evaluation samples were also analyzed by RP-HPLC, as described (16).

**Fig. 1.** Quaternary forms of seminal RNase before and after tryptic digestion. Panel A, the MxM form, drawn with MOLSCRIPT (38) from PDB file 1BSR (10); Panel B for M–M, constructed from the structure of MxM and from that of the linker peptide of RNase A (39). Panels B–D and F–H are schematic representations of the MxM and the M–M forms, respectively, after 5 (B and F), 15 (C and G), and 20 (D and H) min of tryptic digest. In yellow are represented the intersubunit disulfide bridges that link the two identical subunits, illustrated in green and blue, respectively, to show the interchange of the NH₂-terminal ends, as in MxM and derived products, or lack of interchange, as in M–M and derived products. Intrachain disulfides connecting free fragments are also represented in yellow. Red are indicated the NH₂- and NH₂-, and the COOH- and COOH- terminal ends of each subunit before (A and E) and after (B–D and F–H) tryptic digestion.

in 0.4% ammonium bicarbonate, pH 8.5. The extent of proteolysis was monitored on a time course basis by sampling the reaction mixture at different reaction times ranging from 5 to 20 min. Samples were fractionated by RP-HPLC on a Vydac C18 column; peptides were eluted by means of a linear gradient of 5–40% acetonitrile in 0.1% trifluoroacetic acid over 60 min; elution was monitored at 220 nm. Individual fractions were collected and identified by ESMS (electrospray mass spectrometry, see below). Alternatively, aliquots of the peptide mixtures were analyzed directly by MALDI-MS without any purification step. When necessary, identification of disulfide-bridged protein fragments was carried out by reduction of samples with dithiothreitol in 50 mM Tris-EDTA, 6 M guanidinium chloride, pH 7.5, for 2 h under a nitrogen atmosphere at 37 °C and alkylated with iodoacetamide (Fluka) in the same buffer for 20 min at room temperature in the dark. The modified components were separated by RP-HPLC and analyzed by ESMS.

**Mass Spectrometry**—Proteolytic fragments obtained by limited proteolysis of BS-RNase forms, and separated by RP-HPLC (see above), were analyzed by ESMS using a BIO-Q triple quadrapole mass spectrometer (Micromass) equipped with an electrospray ion source. Samples were directly injected into the ion source (kept at 80 °C) via a loop injection at a flow rate of 10 μl/min. Data were acquired and elaborated using the MASS-LINK program (Micromass). Mass calibration was performed by means of the multiply charged ions from a separate injection of horse heart myoglobin (average molecular mass 16951.5 Da); all masses are reported as average mass.

MALDI mass spectra were recorded using a Voyager DE MALDI-TOF mass spectrometer (PerSeptive Biosystem). A mixture of anolyte solution, α-cyano-4-hydroxycinnamic acid (Sigma) and bovine insulin (Sigma) was applied to the sample plate and dried at room temperature, under atmospheric pressure. Mass calibration was performed using the molecular ions from bovine insulin at 5734.5 Da and the matrix at 379.1 Da as internal standards. Raw data were analyzed by using a computer software provided by the manufacturer and are reported as average masses.

**Interconversion of Dimeric Forms of BS-RNase**—Homogeneous MxM and M–M preparations, obtained as described (11), were incubated at...
form was tested (see Fig. 2, B and C). It has been shown previously that a 17-kDa product is the limit digest product for either MxM or M= M form after a prolonged treatment with trypsin (16).

The intriguing result of all these experiments is that most of the protein remains undigested, even after 2 h of treatment. Because the protein substrates were in large excess (45:1, mol/mol for the dimers; 90:1 for subunits), the possibility was investigated that the concentration of trypsin was limiting. However, identical results of a minimal fraction of protein being digested were obtained when the concentration of trypsin was increased 10-fold (data not shown).

Another possibility was also considered: that in the BS-RNase equilibrium mixture, or in the isolated forms, only a preexisting fraction of each protein was a substrate for trypsin, and most of the proteins were resistant to digestion. But a prolonged incubation of BS-RNase under identical digestion conditions, illustrated in Fig. 2D, shows that all of the incubated protein would be digested eventually.

These results led to the hypothesis that the real, limiting substrates of trypsin were not the MxM and the M= M form after a prolonged treatment with trypsin (16).

The kinetics of MxM or M= M depletion in the presence of trypsin was thus investigated. MxM and M= M forms (38 μM)
Trypsin and Seminal RNase Dimeric Forms

Electrospray mass spectrometric identification of peptide fragments generated during tryptic hydrolysis of the quaternary forms of BS-RNase, MxM, and M–M, and of its monomeric derivative M–Cm

The NH2- and the COOH-terminal ends of the peptide fragments are indicated within parentheses when the fragments are linked by single or double disulfide bridges. To distinguish the two subunits, the sequence positions of one of them are denoted with prime superscripts at the indicated sequence positions.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Molecular mass (Da)</th>
<th>Identification</th>
<th>Molecular mass (Red-Cam) (Da)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>MxM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>27,201.7</td>
<td>1–124 (1–124)</td>
<td>14,182.8</td>
<td>1–124 (10 Cam-Cys)</td>
</tr>
<tr>
<td>5</td>
<td>1,135.8</td>
<td>1–10</td>
<td>14,183.5</td>
<td>1–124 (10 Cam-Cys)</td>
</tr>
<tr>
<td>15</td>
<td>20,641.5</td>
<td>(11–124) (1–55)–(81–91)</td>
<td>13,063.9</td>
<td>11–124 (10 Cam-Cys)</td>
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<td></td>
<td></td>
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<td>11–124 (10 Cam-Cys)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>6,342.2</td>
<td>1–55 (4 Cam-Cys)</td>
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<tr>
<td>M=M</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>0</td>
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<td>1–124 (1–124)</td>
<td>14,183.0</td>
<td>1–124 (10 Cam-Cys)</td>
</tr>
<tr>
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<td>1,135.8</td>
<td>1–10</td>
<td>14,182.9</td>
<td>1–124 (10 Cam-Cys)</td>
</tr>
<tr>
<td>15</td>
<td>20,640.8</td>
<td>(1–124) (11–55)–(81–91)</td>
<td>14,184.3</td>
<td>1–124 (10 Cam-Cys)</td>
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<td></td>
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<tr>
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<td>1–124 (10 Cam-Cys)</td>
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<tr>
<td></td>
<td>6,484.3</td>
<td>(11–33)–(81–98)–(40–55)</td>
<td>3,396.8</td>
<td>11–39 (3 Cam-Cys)</td>
</tr>
</tbody>
</table>

* Molecular mass values of the peptides identified after selective reduction of disulfide bonds followed by sulphydryl carboxamidomethylation (Cam).
* Carboxyamido-methylated (Cam) or carboxymethylated (Cm) cysteines.
* Peptides detected by MALDI-MS analysis.

were incubated separately for 0–21 h at 37 °C in Tris acetate, pH 8.4, with 0.9 μg trypsin. The kinetics data could be fitted to pseudo-first-order plots with satisfactory correlation coefficients (≥ 0.99), from which the rate constants \( k_{MxM} = 2.1 \times 10^{-4} \) min⁻¹ and \( k_{M=M} = 4.7 \times 10^{-4} \) min⁻¹ were determined for the MxM and the M–M form, respectively. These constants could be related to the rates of tryptic hydrolysis of the intermediate forms susceptible to tryptic attack or to the rate-limiting formation from the MxM and M–M forms of their respective conformational intermediates. The latter interpretation appears as the only realistic one, based on the following considerations. (i) The rates measured for MxM and M–M are 2–3 orders of magnitude lower than those reported in the literature for tryptic hydrolysis of proteins, folded or unfolded (21, 22). Furthermore, when a preparation of S protein dissociated from S peptide was treated with trypsin under the same conditions employed for MxM and M–M, a value of \( k_{\text{hydrolysis}} = 1.240 \times 10^{-4} \) min⁻¹ was measured. RNase S, the complex of fragments 1–20 (S peptide) and 21–124 (S protein), is generated by limited proteolysis of RNase A with subtilisin (23). The S protein thus isolated, in which the NH₂-terminal arm is missing, is a close analog to a BS-RNase subunit in which the NH₂-terminal arm has been dislocated. (ii) The rates were insensitive to increases of trypsin concentration (see above), (iii) The first peptide bond cleaved by trypsin (see below) is the same (Arg₁₀–Gln₁₁) when either MxM or M–M is subjected to tryptic degradation; this indicates that the microscopic substrate of trypsin is identical in either form when they are treated with trypsin.

It can thus be concluded that the values of 4.7 and \( 2.1 \times 10^{-4} \) min⁻¹ are the rate constants of formation from the M=M and the MxM forms of their respective conformational intermediates. This conclusion, and specifically the slower formation of intermediate in MxM, hence its lower propensity to undergo a conformational change, is perfectly in line with the higher stability of MxM at equilibrium. In fact the ratio \( k_{M=M}/k_{MxM} \) of about 2.3 between the constants of formation of intermediates approximates the equilibrium ratio [MxM]/[M–M] of about 2 in the native protein (11).

Sequential Steps of Tryptic Action on the Quaternary Forms of BS-RNase—The action of trypsin on the isolated forms of BS-RNase was determined step by step by mass spectrometric analyses on homogeneous M=M or MxM forms (38 μm) digested with trypsin as described under "Experimental Procedures." The chronological sequence of tryptic cleavages was deduced from the identification of tryptic products on a time course basis. Aliquots of each incubation mixture were fractionated by RP-HPLC (see "Experimental Procedures") before and after reduction of disulfides with dithiothreitol, followed by alkylation with iodoacetamide of the exposed sulphydryls. The separated fragments were identified in the amino acid sequence of BS-RNase subunit by mass spectrometric analyses.

In Table I the identified fragments are tabulated. At the shortest time interval (5 min) the molecular mass values of the large digestion products were not determined directly, as these
products coeluted with undigested MxM or M-M. However, these values could be determined after reduction and alkylation of the intrachain and interchain disulfides of the fragments, and from the detection by MALDI-MS of the smaller excised fragments. Strikingly, at each time interval identical mass values were determined upon digestion of MxM or M-M, but after disulfide cleavage and sulfhydryl alkylation, different values for the identified fragments were obtained (see Table I).

The most relevant result is that for both forms the first tryptic nick, registered at 5 min, occurs at Arg10 of one of the subunits in each dimer. At 15 min only the nicked subunit in the M-M form, whereas only the intact subunit in the MxM form, is cleaved further at several sites. At 20 min the limit digest product of 17 kDa is generated for each form.

These results clarify the previous finding (16) of a different limit digestion product for the two quaternary forms. The cleavage sites are identical in the two forms, but on different subunits, and cleavages occur in an identical time succession, thus the intermediate and final digestion products are not identical for the two forms. As shown in Fig. 1, F and G, in the M-M form after the first cut at Arg10, the attacked subunit is degraded progressively until only its 11–39 segment remains; this is linked by the intersubunit disulfides with the other subunit, which remains entirely resistant to trypsin. For the MxM form instead (Fig. 1, B and C) it is the nicked subunit, now missing peptide 1–10, which remains resistant to trypsin, whereas the partner subunit is degraded down to its 1–39 fragment, also linked to the partner chain by the intersubunit disulfides.

These unequal degradation effects of trypsin on the two forms are entirely the result of the main structural feature of each form: the exchange (MxM) or lack of exchange (M-M) of the NH2-terminal arm between subunits. Upon scission of the Arg10-Gln11 bond, the proteolytic removal of the 1–10 arm in one subunit of the MxM form exposes most of the potentially susceptible tryptic sites in the partner subunit, whereas in the M-M form, the removal of the 1–10 arm exposes the available sites on the same nicked subunit.

The finding that the tryptic cleavages occur (both in MxM and M-M) only in one subunit may appear surprising, but it is likely that the binding of one trypsin molecule to one Arg10 site in one subunit hinders the binding of a second trypsin molecule.

The Action of Trypsin on Monomeric BS-RNase—A stably monomeric, enzymatically active derivative of seminal RNase (termed M-Cm) is obtained by selective reduction of its inter-subunit disulfides followed by carboxymethylation with iodoacetic acid of the exposed sulfhydryls (18). When this monomeric derivative at a concentration of 36 μM was tested with trypsin (0.2 μM), it was found to be resistant under the conditions (see “Experimental Procedures”) in which trypsin was active on BS-RNase or on its isolated forms. When the trypsin concentration was increased to 0.8 μM, tryptic degradation of M-Cm was detected by mass spectrometric analyses, but with a succession of cleavages radically different from that registered for MxM or M=M (see Table I). The first detectable cleavage sites, as determined by mass spectrometric analyses after 10 min of incubation, were in the main body of the protein. They were generated by rapid and virtually simultaneous cuts at several sites, including those of lysine 55, 61, and 104. After a longer incubation, further cuts were revealed at arginine 80 and 33, and at lysine 98. The cleavage at arginine 33 is found only in the degradation of M-Cm and not in the dimeric forms of BS-RNase (see above). This is apparently because of its location at the surfaces hidden in the dimers at the intersubunit interface; these surfaces become exposed in the monomers.

It may be surprising that a less stable form of BS-RNase, such as its monomeric derivative M-Cm (24, 25), is more resistant to trypsin than the more stable dimeric forms of the protein. The explanation for this apparent discrepancy can be found in the other more relevant result from these experiments: the lack of cleavage of the bond Arg10-Gln11 in the initial phase of tryptic digestion of M-Cm. We propose that the resistance of the M-Cm monomeric form of BS-RNase at lower trypsin concentrations is not caused by a higher stability of the monomer as a whole, but rather by a higher stability of the NH2-terminal arm, which is not dislocated as it occurs in the dimeric forms, hence it is not cleaved at Arg10. This conclusion is also supported by previous results (26) on the inability of M-Cm to associate into dimers effectively, even after lypophilization from acetic acid solutions, as RNase A does through the interchange of the NH2-terminal arm between monomers (3).

The Action of Trypsin on Dimeric RNase A—Native, monomeric RNase A is resistant to tryptic hydrolysis up to a temperature of 60 °C (27). The quaternary structure of RNase A dimers produced by lypophilization from acetic acid (3) is maintained through the exchange between protomers of their NH2-terminal arms (4, 28, 29). Dimeric RNase A is metastable (19) and has a quaternary structure different from that of MxM and M=M (4). However, it shows with the MxM form of seminal RNase the interchange of NH2-terminal arms between subunits, an 80% identity in amino acid sequence, and an identical Arg10-Gln11 peptide bond.

Dimeric RNase A (13 μM) was treated at 37 °C with 0.3 μM trypsin in 0.1 M Tris acetate at pH 8.4 containing 0.27 M NaCl or in 0.2 M sodium phosphate at pH 6.5. After 1–7 h of digestion, aliquots were withdrawn, the reaction was stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM, and the digestion mixtures were analyzed by RP-HPLC. In contrast with monomeric native RNase A, dimeric RNase A was found to be digested by trypsin into a broad number of fragments. The digestion pattern was similar (data not shown) to that obtained with denatured RNase A (30). By a preliminary mass spectrometric analysis, several of the digestion products expected from an extensive digestion of the protein were identified (data not shown). In particular, the digestion was found to be slower when the incubation buffer was phosphate, a buffer in which the stability of the dimers is higher (19).

These data indicate that when associated into unstable dimers, RNase A becomes susceptible to tryptic hydrolysis, but this may not be considered a case of limited proteolysis, as most if not all cleavable bonds are cleaved readily. Hence trypsin degrades dimeric RNase A in a totally different fashion from that observed for a similar dimer such as MxM.

Nonetheless, the comparison of the effects of trypsin on dimeric RNase A with those observed on the MxM form of BS-RNase is still instructive. Although both unstable, dimeric RNase A and MxM have different fates in their approaches to equilibrium. Dimeric RNase A dissociates into monomers (19), and it does so through an unfolding step that makes the protein as a whole susceptible to extensive tryptic digestion. This is shown by the absence of an initial cleavage at Arg10. MxM instead unfolds only in its NH2-terminal region, and an immediate cleavage at Arg10 can occur. In fact only upon removal of its NH2-terminal peptide segment does a deprived BS-RNase subunit become as a whole susceptible to trypsin.

A Model for the Interconversion MxM ⇔ M=M in Seminal RNase—The first, basic consideration is that both the MxM and M=M forms of seminal RNase may not be considered as stable protein structures. When separated from each other, each form readily sets to transform into the other until equilibrium is established, and native seminal RNase is an equi-
Trypsin and Seminal RNase Dimeric Forms

Fig. 4. A model for the interconversion of BS-RNase quaternary forms MxM and M=M. Mxl, I=I, and M=I represent intermediates in the interconversion process with one or both subunits with I conformation, i.e., with a displaced NH₂-terminal arm(s). The arrows indicate the sites of the first tryptic cleavage in the displaced NH₂-terminal arms of the intermediates Mxl or M=I nearest to the MxM and the M=M form, respectively.

A mixture of the two forms and of all intermediate equilibrium forms. Hence a realistic description of seminal RNase must be dynamic and represent it as two protein conformations undergoing a continuous unfolding and refolding of structural parts, the NH₂-terminal arms. Thus the first immediate necessary intermediates in the two kinetic pathways, or the equilibrium intermediates respectively nearest to the MxM or M=M form, are dimeric forms in which the NH₂-terminal arm is unfolded and dislocated from the main body of one of the two subunits. If the subunit with an unfolded arm is termed “I,” these intermediates will be Mxl and M=I, respectively, nearest to MxM and M=M. In the species undergoing a complete conversion, the subsequent steps would consist in: first, the same dislocation occurring also in the partner subunit and then in the repositioning of the dislocated arms onto different subunits. The two kinetic pathways and the equilibrium can be described as shown below.

\[ M = M \Leftrightarrow M = I \Leftrightarrow I = I \Leftrightarrow IxM \Leftrightarrow MxM \]

**REACTION 1**

This description is in line with the results reported in this paper. In the structure of seminal RNase (10), Arg₁⁰ is on the inaccessible face of the 3–13 α-helix and is salt-linked with Glu₂. As such, it could not bind to the trypsin active site. The finding that cleavage does occur at Arg₁⁰ may only indicate that the salt link Arg₁⁰-Glu₂ is disrupted and that the 3–13 α-helix is unfolded. It has been shown that proteolysis is highly unlikely to occur on a folded, rigid α-helical protein segment (31). Thus any species containing a subunit in the I conformation can be a substrate for trypsin (see Fig. 4). Because Mxl and M=I are the first intermediates to be formed in the conversion pathways, their removal by tryptic hydrolysis will stop the conversions (see Fig. 3).

The Mxl, M=I, and I=I intermediates may not be defined in individual molecular terms, as they are topologies of intermediates, given the length and complexity of the itineraries of the NH₂-terminal arms moving from one subunit to another. However, the ability of trypsin to identify through degradation the first unfolded species, and the rate-limiting property of the unfolding step of the NH₂-terminal arm, give the opportunity to identify kinetically the two respective Mxl and M=I intermediates. The finding that the M=M \rightarrow M=I step is faster than the MxM \rightarrow Mxl step is also in line with the higher stability of the MxM form, a structure that shares with M=I one interface but has one additional interface formed through the exchange of the NH₂-terminal arms (see Fig. 1).

The model as proposed is also in line with literature data (31, 32) pointing to the importance of specific features of the sites susceptible to limited proteolysis, such as accessibility, flexibility, and lack of secondary structure. A detailed comparison of the results reported here with those predicted by the algorithm derived by Hubbard et al. (32) will be presented elsewhere.²

**Significance of the Dual Quaternary Structure of Seminal RNAse**—In the *in vitro* refolding pathway of fully denatured and reduced BS-RNase, folded monomers form first, then they associate into the M=M dimeric form; the M=M form however is not stable as such, and it converts slowly into the MxM form until equilibrium is established (11, 33). The instability of the M=M form is intriguing, as this is a conformation reached at the end of a folding pathway, a conformation in which the enzyme is fully active (33). This instability may be related to the lack of a single deep energy minimum for the M=M conformation, which in turn can be dependent on the observed lack in M=M of a single stable conformation for the hinge peptides connecting each NH₂-terminal arm to its subunit (12). Thus M=M can easily enter the conversion pathway, and the dimer fraction reaching the end of the pathway is trapped in the more stable MxM conformation.

The instability of the MxM form is also intriguing; it is more stable than M=M but not stable enough to be the only conformation of BS-RNase. As a metastable dimer, MxM is reminiscent of other metastable dimers in which the quaternary structure is maintained similarly by the exchange of structural parts between subunits. These include the dimers of RNase A (3, 4), of the CD2 lymphocyte cell adhesion molecule (5), of diphtheria toxin (6), and of barnase (7). These dimers are found to dissociate into monomers unless frozen in a crystal structure, and their monomeric conformations represent their respective stable conformations. It is proposed that the MxM dimeric form of seminal RNase is as metastable as the other swapped dimers and that the unfolding of its NH₂-terminal arm(s) is the first step toward monomerization. But MxM cannot dissociate into monomers as the other dimers do because its dimeric structure is maintained also by covalent cross-links, the intersubunit disulfides. On the other hand, the dislocation of the NH₂-terminal arms, the first step toward monomerization, will direct the subunits toward the M=M conformation. Thus in equilibrium seminal RNase we do not find only the nascent form of the dimeric protein, i.e., the metastable dimer M=M, as this in part transforms into a more stable dimer (MxM), which tends toward the most stable form for that polypeptide chain, the monomeric form, the evolutionary preferred structure for an RNase of the same superfamily (9).

This intriguing phenomenon may in fact find an explanation in evolutionary terms: seminal RNase could be an evolutionary experiment yet to be completed. All of the known members of the superfamily of pancreatic-type RNases (9) are monomeric proteins, except for seminal RNase. Whereas all genes encoding monomeric RNase have a much longer evolutionary history, the seminal-type RNase gene is only about 5 million year old, the age of *Bos taurus* (34), the only known organism with a seminal ribonuclease expressed gene (35). It should also be noted that the two forms of seminal RNase are different not only structurally but also functionally. The MxM form of the enzyme displays properties absent in the M=M form, such as the allosteric regulation of the hydrolytic reaction step (36) and a more powerful cytotoxicity (13–15). It may thus be envisioned that evolution is still experimenting with this protein, both with its structure and its function, and hence be concluded that the data reported here on the interconversion of seminal RNase dimeric forms are in line with the earlier suggestion that seminal RNase is a case of evolution in progress (37).

² M. V. Cubellis and G. D’Alessio, manuscript in preparation.
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