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Selective and asymmetric action of trypsin on the dimeric forms of seminal RNase

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Abstract

Dimeric seminal RNase (BS-RNase) is an equilibrium mixture of conformationally different quaternary structures, one characterized by the interchange between subunits of their N-terminal ends (the MXM form); the other with no interchange (the M=M form). Controlled tryptic digestion of each isolated quaternary form generates, as limit digest products, folded and enzymatically active molecules, very resistant to further tryptic degradation. Electrospray mass spectrometric analyses and N-terminal sequence determinations indicate that trypsin can discriminate between the conformationally different quaternary structures of seminal RNase, and exerts a differential and asymmetric action on the two dimeric forms, depending on the original quaternary conformation of each form. The two digestion products from the MXM and the M=M dimeric forms have different structures, which are reminiscent of the original quaternary conformation of the dimers: one with interchange, the other with no interchange, of the N-terminal ends. The surprising resistance of these tryptic products to further tryptic action is explained by the persistence in each digestion product of the original intersubunit interface.

Keywords: BS-RNase; domain swapping; limited proteolysis; mass spectrometry; trypsin

Bovine seminal RNase (BS-RNase) is an RNase with unusual biological actions, and the only dimeric member of the vertebrate ribonuclease superfamily (D'Alessio et al., 1997). The crystallographic analysis of its structure determined at 1.9 Å resolution (Mazzarella et al., 1993) has shown that: (1) as expected from the high similarity in amino acid sequence (more than 80%), the two identical subunits have a structure very similar to that of RNase A; (2) the two subunits interchange their N-terminal helical segments, giving rise to an intertwined or domain-swapped (Schlunegger et al., 1997) quaternary structure. This structure has been termed the MXM dimeric form of seminal RNase, because a quaternary structure with no interchange of parts (the M=M form) has also been described for the protein (Piccoli et al., 1992). The native enzyme is, in fact, an equilibrium mixture of MXM and M=M dimers, in a molar ratio of 2:1.

As a dimer, BS-RNase is rather resistant to proteases, including trypsin and subtilisin (Parente et al., 1976). Monomeric, stable, and active derivatives of BS-RNase, although resistant to subtilisin, are readily degraded by trypsin (Parente et al., 1976). Although selectively cleaved by subtilisin under mild conditions (Richards & Vithayathil, 1959), RNase A is also resistant to trypsin and thermolysin, unless denatured by thermal treatment or with trifluoroethanol; in these conditions RNase segments 31–39 and 41–48 in the amino acid sequence become susceptible to enzymatic cleavage (Arnold et al., 1996; Polverino de Laureto et al., 1997).

Here we report that under suitable conditions the two forms of dimeric BS-RNase are both selectively hydrolyzed by trypsin, but with a different bond selectivity. The tryptic digestion of the isolated quaternary forms MXM and M=M generates folded and stable limit digestion products, endowed with high RNase activity.

Results

When BS-RNase (i.e., the natural 2:1 mixture of MXM and M=M) was incubated with trypsin for 2 h at 37 °C in 0.1 M Tris-acetate buffer, pH 8.4, using an enzyme to substrate ratio of 1:50 (w/w),

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Abbreviations: BS-RNase, bovine seminal RNase; CM-Cys, carboxymethyl-cysteine; ESMS, electrospray mass spectrometry; HPLC, high-performance liquid chromatography; MXM, dimeric form of BS-RNase with exchange of N-terminal ends between subunits; M=M, dimeric form of BS-RNase with no exchange; 17K(MXM) and 17K(M=M), 17 kDa stable products of tryptic digestion of the MXM and M=M dimeric forms of BS-RNase, respectively; PMSF, phenylmethylsulfonyl fluoride; RNase A, bovine pancreatic RNase (EC.3.1.27.5); SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TFA, trifluoroacetic acid.

most of the protein remained unmodified, but a degradation product, with an apparent molecular mass of 17 kDa, was detected by SDS-PAGE (Fig. 1). This product was also present when a trypsin inhibitor such as phenylmethylsulfonyl fluoride (PMSF) was added to the reaction mixture to stop the proteolytic reaction (data not shown). This indicates that the 17 kDa product was generated during the tryptic reaction, rather than in the denaturation step preceding the electrophoretic analysis. When the digestion time was increased to 20 h, the 17 kDa product significantly increased (see Fig. 1), indicating that this is a stable tryptic product of BS-RNase, which tends to accumulate. After 72 h of hydrolysis about 50% of the dimeric protein was found to be degraded.

Identical results were obtained when the isolated MXM and M=M dimeric forms of BS-RNase were treated with trypsin under the same conditions (data not shown).

The isolation and characterization of the 17 kDa tryptic products from the MXM and the M=M forms of seminal RNase

To prepare the limit digest products of each BS-RNase dimeric form, the isolated forms were subjected to tryptic hydrolysis for 72 h under the conditions described above. The two hydrolyzates were then fractionated by reverse-phase HPLC (data not shown), and the individual components analyzed by ESMS.

In the hydrolyzates from either quaternary form, the main components were found to be the 17 kDa product and the undigested dimeric protein. (A detailed ESMS analysis of all the smaller peptide fragments produced by the tryptic digestion of the MXM and M=M forms, as well as the digestion time course, will be described elsewhere.) The latter was identified from its observed molecular mass of 27,201.3 ± 1.0 Da, coincident with that calculated for native dimeric BS-RNase, containing eight intrasubunit and two intersubunit disulfide bridges (27,201.0 Da). Its N-terminal sequence (residues 1–4), determined by Edman degradation, corresponded to that of native BS-RNase.

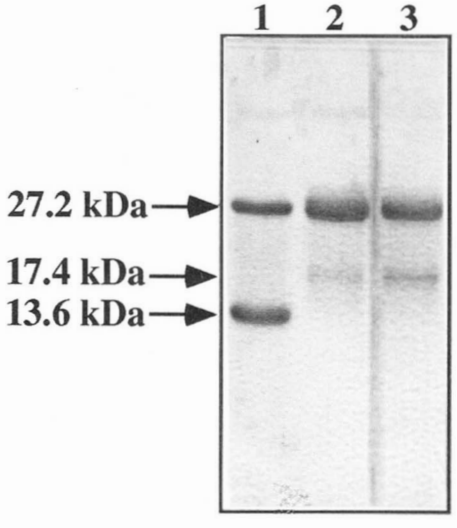

Fig. 1. SDS-PAGE of BS-RNase (10 μg) digested with trypsin at 37 °C for 2 h (lane 2) or 20 h (lane 3). BS-RNase and RNase A (lane 1) were used as molecular-weight standards (molecular weights: 27,200 and 13,600 Da, respectively).

The ESMS analysis of the 17 kDa limit digest product from the MXM form [henceforth termed 17K(MXM)] revealed a molecular mass of 17,431.7 ± 0.7 Da, as shown in Figure 2A. The same analysis, carried out for the product from the M=M form of BS-RNase [henceforth termed 17K(M=M)], is illustrated in Figure 2B. Its measured molecular mass (17,431.4 ± 0.9 Da) was coincident with that determined for 17K(MXM).

N-terminal sequence analyses of the 17K(MXM) and 17K(M=M) tryptic products were performed by the Edman reaction. For both products, the simultaneous release of three residues, as phenyl-thioidantoin derivatives, at each Edman degradation cycle, was observed. This suggested that both molecules consisted of three fragments held together by disulfide bridges. The N-terminal sequences of these fragments, reported in Table 1, were identical for both 17 kDa products. On the basis of the BS-RNase subunit sequence, they were respectively identifed as: the N-terminal sequence of the subunit; the N-terminal sequence starting from Gln11, produced by a tryptic cleavage at Arg10; and the N-terminal sequence from Ile81, following a tryptic cleavage at Arg80.

These data led us to conclude that the two limit tryptic products, 17K(MXM) and 17K(M=M), with identical mass and identical amino acid composition, are structurally identical. However, the ESMS analyses of the two products after disulfide reduction, followed by alkylation of the exposed sulfhydryls, revealed that this is not the case.

Fully reduced and alkylated 17K(MXM) and 17K(M=M) were each separated by HPLC (data not shown). Two main fragments were obtained from each product: components A and B from 17K(MXM); and C and D from 17K(M=M). The ESMS analysis of component A showed a molecular mass of 13,074.3 ± 1.1 Da, which corresponds to the fragment 11–124 of BS-RNase subunit carrying 10 carboxymethyl-cysteines (CM-Cys). For component B a mass value of 4,518.2 ± 0.9 Da was measured, which led to its identification with the fragment 1–39 of the subunit chain of BS-RNase with three CM-Cys residues. The fragment 81–85 was undetectable, apparently eluted in the void volume of the HPLC column, due to its low molecular mass and high polarity.

These interpretations were confirmed by Edman sequencing of the two fragments. The N-terminal sequence of component A (Gln-His-Met-Asp) corresponded to that of residues 11–14 of the BS-RNase subunit chain; component B showed an N-terminal sequence (Lys-Glu-Ser-Ala) coincident with that of the intact BS-RNase subunit chain.

These data, and those reported above from Edman sequencing and ESMS analyses before reduction and alkylation of the tryptic product from the MXM form, led to the elucidation of the complete primary structure of 17K(MXM), illustrated in Figure 3A.

The ESMS analyses of fragments C and D from reduced and alkylated 17K(M=M) produced the following results. Component C displayed a molecular mass of 14,181.4 ± 1.2 Da, which corresponds to an intact BS-RNase subunit chain (1–124) containing 10 CM-Cys residues. Component D showed a mass value of 3,396.8 ± 0.2 Da and was identified with the peptide 11–39 of the subunit chain carrying three alkylated cysteines. The primary structure of 17K(M=M) is illustrated in Figure 3B.

Thus, the data reported above demonstrate that tryptic hydrolysis of the isomeric BS-RNase forms produced two different products that share the same molecular mass but have different structure. Both 17 kDa products consist of partially digested BS-RNase having two larger chain fragments held together by the two intersubunit disulfides of the original dimer and generated by asymmetric

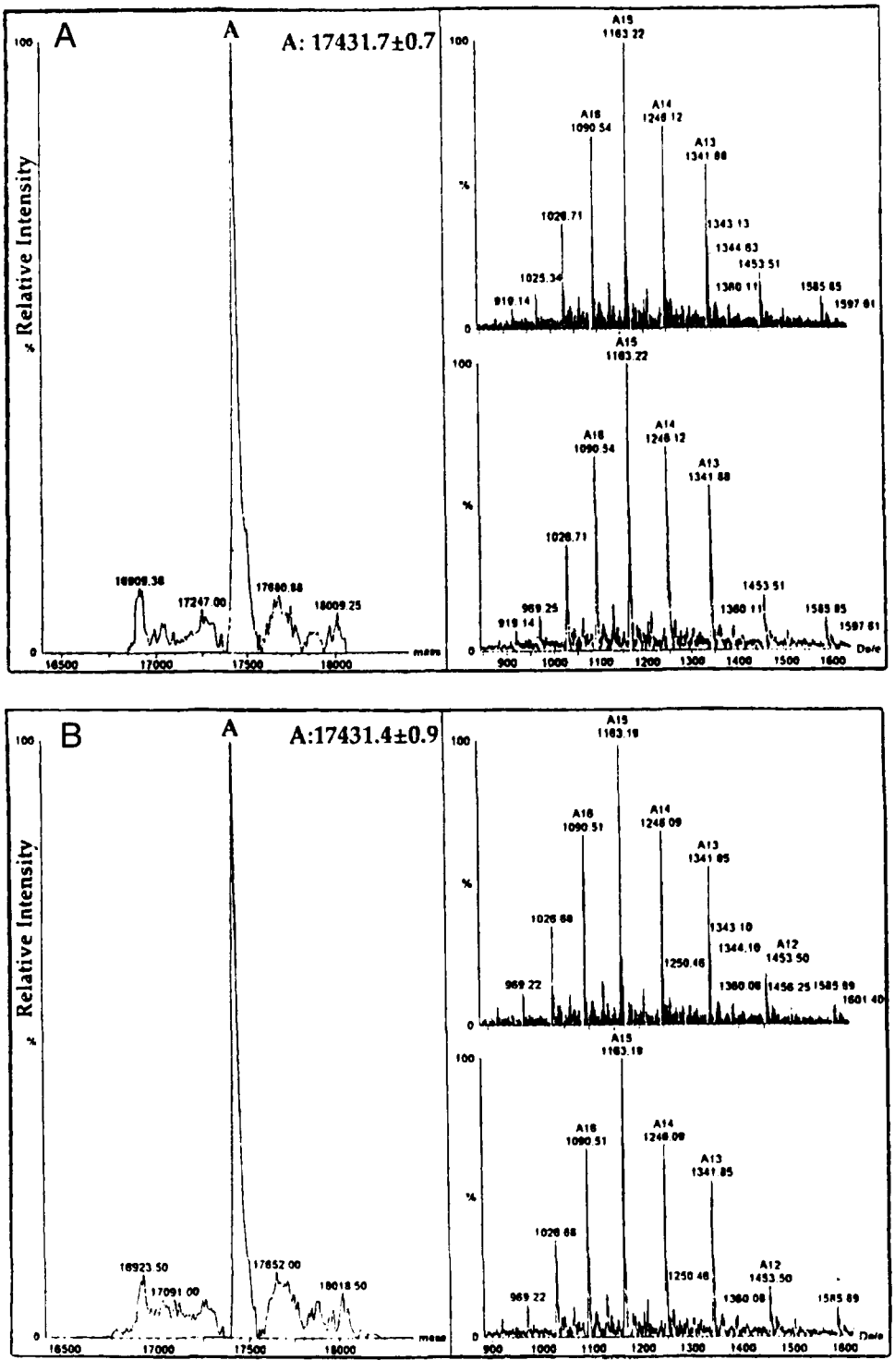

Fig. 2. Electrospray mass spectrometric analysis of (**A**) 17K(MXM) and (**B**) 17K(M=M) products from tryptic hydrolysis of BS-RNase quaternary forms. The products, purified by HPLC, were directly injected into the ion source of the mass spectrometer. Both the multiply charged ions and the transformed spectra are shown. The measured molecular mass values are indicated.

highly specific cleavage of protein segments from the two subunits of the native homodimer. The 17K(MXM) tryptic product is formed by a larger chain, corresponding to the fragment 11–124 of the BS-RNase subunit. It contains all the intrasubunit disulfide bridges present in the native protein and is linked to a fragment (1–39) from the partner subunit, which in turn, is linked to fragment 81–85 by the disulfide bridge between Cys26 and Cys84. The 17K(M=M) tryptic product possesses a similar but distinct struc-

Table 1. *N-terminal amino acid analysis by the Edman reaction of the 17K(MXM) and 17K(M=M) products*

Edman cycle			
1	2	3	4
N-terminal residues			
Lys	Glu	Ser	Ala
Gln	His	Met	Asp
Ile	Thr	Asp	—
Identified segments in BS-RNase sequence			
Lys1-Glu-Ser-Ala4			
Gln11-His-Met-Asp14			
Ile81-Thr-Asp83			

ture, in which the larger chain corresponds to an entire subunit of BS-RNase (1–124), whereas from the partner subunit only fragments 11–39 and 81–85 subsist, held together by the Cys26–Cys84 disulfide.

Both isolated 17K(MXM) and 17K(M=M) products were found to be very resistant to further tryptic cleavage. When incubated at 37 °C with trypsin in 0.1 M Tris-acetate buffer at pH 8.4, using an enzyme to substrate ratio of 1:50 (w/w), only 3–5% of either product was found to be degraded in 24 h of incubation.

Functional and structural features of the 17K(MXM) and the 17K(M=M) tryptic products

The enzymatic activity of the limit digest products of tryptic hydrolysis of BS-RNase dimeric forms was tested by the Kunitz assay on yeast RNA as a substrate (Kunitz, 1946). Although extensive parts of each form were removed by trypsin, the activity of both products (30–35 Kunitz U/mg) was found to be comparable to that of native BS-RNase.

Monomeric derivatives of BS-RNase are more active than the dimeric native enzyme (D'Alessio et al., 1975); thus, the lower activity (with respect to a monomer) of the 17K(MXM) product may be due to the non-native structure of its active site. The lack of an increase in enzymatic activity of 17K(M=M), which contains an intact subunit, remains unexplained.

Because the two tryptic limit products derive from two different quaternary structures, one in which the two subunits interchange their N-terminal α-helices, and one in which each chain folds independently, the question was addressed: are the two tryptic products reminiscent of the two respective original quaternary structures? To answer this question, the two digestion products were treated with an aminopeptidase (see Materials and methods). At 24 h intervals, aliquots were withdrawn from each sample, and the N-terminal sequences were analyzed by Edman degradation (three cycles).

After 96 h of incubation, we found that in both samples the N-terminal sequences of the fragments starting with Lys1 (i.e., fragments 1–39 of 17K(MXM) and 1–124 of 17K(M=M), see Fig. 3A,B) were unaltered, as expected from the specificity of the aminopeptidase, which does not attack N-terminal Lys residues. Three N-terminal residues were instead found to be removed by the aminopeptidase from the fragments starting with Gln11, i.e., fragments 11–39 of 17K(M=M) and 11–124 of 17K(MXM): Gln11, His12, and Met13. The enzymatic activity of the *des*(11–13)-17 kDa products was then tested on yeast RNA (Kunitz, 1946). The results indicated that after the removal of the three residues, both derivatives retained about 90% of their original activity.

This finding may not be relevant for the 17K(M=M) product, which contains a full, complete active site in its intact monomer moiety. The finding is instead relevant for the 17K(MXM) product. In the latter case, the persistence of enzymatic activity indicates that the catalytically essential His12 is contributed by fragment 1–39, whereas the other essential His119 is in fragment 14–124. This leads to the conclusion that 17K(MXM) has a composite active site, comprised of histidine residues from two chains, just as in the original structure of the MXM form with the exchange of N-terminal segments.

Discussion

Seminal RNase has been found in previous studies to be resistant to tryptic hydrolysis (Parente et al., 1976). This is not surprising, as the protein has a very compact tertiary fold, and RNase A, with a very similar fold, is resistant unless partially unfolded (Arnold et al., 1996; Polverino de Laureto et al., 1997). But the surprising result reported here is that BS-RNase is slowly hydrolyzed by trypsin. Apparently, in the experiments reported earlier (Parente et al., 1976), the assay used to measure tryptic hydrolysis was not sensitive enough for the detection of the small fraction of digestion product. More surprising is the finding that the action of trypsin on the dimer itself, in either the MXM or the M=M conformation, virtually terminates after only two bonds are cleaved, despite the presence in the resistant peptide chains of several Lys and Arg residues. Some of these sites are not susceptible to trypsin hydrolysis, such as the N-terminal Lys, or are only partially cleaved, such as the Lys-Lys or the Arg-Lys bonds (Di Donato et al., 1986). However, one would expect that, once the protein is destabilized by the first tryptic cleavages, all the potentially susceptible bonds would be cleaved.

The results reported above show instead that in either quaternary conformation, once the Arg10–Gln11, and the Lys39–Cys40 bonds are cleaved, folded, and stable structures subsist, quite resistant to further tryptic cleavage, in contrast with the stable and active monomeric forms of the protein, which are readily degraded by trypsin. Furthermore, these structures reflect in their disparity the two original distinct quaternary forms from which they derive. They share an identical mass, and an identical amino acid composition, but have different primary structures. Both dimeric forms of BS-RNase are asymmetrically, but differentially attacked by trypsin in their subunit chains, although the same bonds are cleaved, at Arg10 and at Lys39. In the MXM form, in which the subunits exchange their N-terminal helices, the two bonds are cleaved in different subunit chains, whereas in the M=M form (with no exchange) the two bonds are both cleaved in the same subunit, while the partner subunit survives the tryptic attack as a whole. In both digestion products, a small fragment (81–85 in the sequence) also remains intact, linked by a disulfide to one of the larger fragments.

An explanation can be advanced for the limited proteolysis by trypsin at only two sites of either quaternary form of BS-RNase. In Figures 3C and 3D models of the 17K(MXM) and 17K(M=M) products are proposed, based on the three-dimensional structures of MXM (Mazzarella et al., 1993), and of a model of M=M (Piccoli et al., 1992), respectively. Recent results from an X-ray

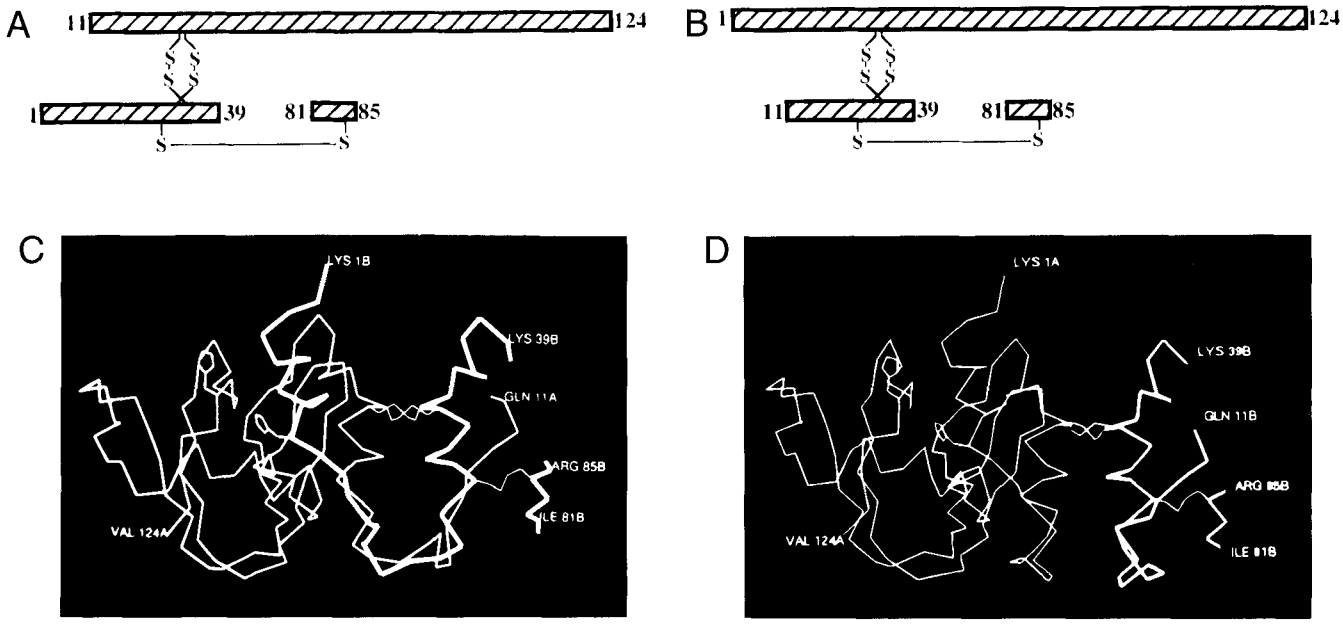

Fig. 3. The structure of the limit tryptic digest products of dimeric BS-RNase forms, (**A, C**) 17K(MXM) and (**B, D**) 17K(M=M). (**A, B**) A schematic view of the primary structures; (**C, D**) models of the three-dimensional structures. The N- and C-terminal residues of BS-RNase A- and B-chain fragments left undegraded by trypsin, and the side chains of the catalytically active His residues, are indicated.

crystallographic analysis of the M=M form of BS-RNase are in line with the latter model (L. Mazzarella, pers. comm.). The structures of the tryptic products of BS-RNase dimeric forms have been constructed by simply removing from the original MXM and M=M forms the segments cleaved by trypsin. From these illustrations it clearly appears that the two digestion products are a monomer [in the case of 17K(M=M)], and a monomer equivalent [in that of 17K(MXM)] of BS-RNase, but both of them are equipped with a perfectly conserved intersubunit interface. In both native dimeric forms of BS-RNase, the interface is formed by the helical segments of the two subunits comprising residues 24–34, and includes the intersubunit disulfides (formed by Cys31 and Cys32) and the hydrophobic interactions (produced by Leu28 and Met29), which hold the two subunits together (Mazzarella et al., 1993). These segments of the original dimeric structures remain undegraded by trypsin and are present in both the 17K(MXM) and the 17K(M=M) tryptic products (see Fig. 3).

The preservation in the two tryptic products of BS-RNase of the original intersubunit interfaces may explain why these structures are exceptionally resistant to further tryptic attacks, whereas monomeric BS-RNase is readily and completely degraded by trypsin (Parente et al., 1976). This conclusion may also be based on the reported findings that in mildly denatured RNase A the surface first attacked by trypsin is that which corresponds to the intersubunit interface in BS-RNase (Arnold et al., 1996).

In the native MXM form, a second intersubunit interface forms between the exchanged N-terminal α-helix, composed of residues 3–13 and the main body of the partner subunit (Mazzarella et al., 1993). This interface is conserved also in the 17K(MXM) product, as indicated by the finding that in this molecule the two catalytically essential His residues are contributed by distinct chains.

As for the asymmetry in tryptic action on the two subunit chains of either form of BS-RNase, it can be hypothesized that in both dimeric forms the first cleavage occurs at Lys39, in the small 35–40 loop connecting the central helix with the first β-strand in the BS-RNase subunit. This loop is partially exposed, and displays a higher temperature factor than the surrounding regions; more importantly, in this loop small differences have been detected between the two chains (Mazzarella et al., 1993). Thus, it can be surmised that the cleavage in one chain is favored for a higher flexibility, hence, a higher affinity of the 35–40 loop in that chain to the trypsin active site. It should again be underlined that in partially denatured RNase A the segment 31–39 is the first susceptible region to limited proteolysis by trypsin and thermolysin (Arnold et al., 1996; Polverino de Laureto et al., 1997). The tryptic cleavage at Lys39 in the MXM form would destabilize the whole remaining 40–124 fragment of that chain, which would therefore be promptly digested into small fragments, except for the segment 81–85, anchored to the 1–39 fragment by a disulfide. The removal of most of one chain could, in turn, destabilize the other chain, in particular its N-terminal α-helix, which would render the bond Arg10–Gln11 susceptible to tryptic hydrolysis.

As for the equally asymmetric tryptic attack on the M=M dimeric form, in which each chain folds onto itself independently from the partner chain, it may be proposed that the tryptic hydrolysis at Lys39 destabilizes the whole subunit, as in an isolated monomer, including the N-terminal segment; only the surface protected through the intersubunit contacts is preserved.

The alternative assignment of the first tryptic cleavage to Arg10 would appear as unlikely: Arg10 is in the N-terminal α-helical segment, which, as a secondary structure element, would be rigid and resistant to proteolysis (Fontana et al., 1997). Furthermore, as in RNase A (Richards & Wickoff, 1971), it is H-bonded to Glu2 (Mazzarella et al., 1993). Only a destabilization of the N-terminal α-helix could thus make the Arg10–Gln11 bond susceptible to a tryptic attack. On the other hand, the possibility could be consid-

ered that the displacement of the N-terminal α-helix might occur in the interconversion of BS-RNase quaternary forms under equilibrium conditions (Piccoli et al., 1992).

Materials and methods

Materials

BS-RNase was purified as previously reported (Tamburrini et al., 1986). Sequencing grade trypsin, modified by reductive methylation to reduce autolysis, was obtained from Promega (Madison, Wisconsin). BS-RNase quaternary forms (MXM and M=M) were isolated following the protocol described previously (Piccoli et al., 1992). Reagents were purchased from Sigma (St. Louis, Missouri).

Proteolysis of BS-RNase quaternary forms

For analytical purposes the tryptic hydrolysis of BS-RNase (2 mg/mL) was carried out in 0.1 M Tris-acetate buffer, pH 8.4, for 2 or 20 h at 37 °C, using an enzyme to substrate ratio of 1:50 (w/w). To isolate the tryptic limit product from MXM and M=M forms, the proteolytic reaction was extended up to 72 h. The reaction was stopped by adding PMSF to a final concentration of 1 mM or soybean trypsin inhibitor in a 10-fold excess with respect to the enzyme weight. Products were then analyzed by SDS-polyacrylamide (15%) gel electrophoresis (Laemmli, 1970). Proteolytically digested BS-RNase samples were fractionated by RP-HPLC (Gold system, Beckman, Fullerton, California) on a Vydac C18 column (25 × 0.46 cm) (Phase Separation); peptides were eluted by means of a linear gradient of 5–40% acetonitrile in 0.1% trifluoroacetic acid (TFA) over 60 min; elution was monitored at 220 nM.

Mass spectrometry

Proteolytic fragments were analyzed by electrospray mass spectrometry (ESMS) using a BIO-Q triple quadrupole mass spectrometer (Micromass, Manchester, UK) 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 16,951.5 Da); all masses reported are averages.

Reduction and carboxymethylation of limit tryptic products

HPLC-purified tryptic products from MXM or M=M (60 μg) were dissolved in 100 μL of 0.1 M Tris-acetate, pH 8.4, and treated with 1 mM dithiothreitol at room temperature for 30 min under a nitrogen barrier. The exposed sulphydryl groups were then carboxymethylated with 2.5 mM iodoacetic acid or iodoacetamide for 40 min in the dark. The mixture was fractionated by RP-HPLC using a Vydac column C8 (Lab Service, Bologna, Italy), equilibrated in 0.1% TFA. Elution was carried out by a linear gradient of 0–40% acetonitrile in 0.1% TFA over 100 min. The flow rate was 1 mL/min.

N-terminal end degradation

The 17 kDa tryptic products from the MXM or the M=M forms were dissolved to a final concentration of 0.3 mg/mL in 0.1 M Tris-acetate, pH 7, containing 10 μM $ZnSO_4$, and incubated with 50 nM aminopeptidase from *Aeromonas proteolytica* (Sigma). At 24 h intervals, 30 μL aliquots were withdrawn, dried on ProSpin membranes (Perkin-Elmer, Foster City, California) by centrifugation at 3,500 rpm, and analyzed for N-terminal sequence.

Other methods

N-terminal sequence analyses were performed using automated Edman degradation procedure on the Protein Sequencer 473 A (Applied Biosystems, Foster City, California). Repetitive yields were better than 50%. Protein concentration was determined by amino acid analysis and a colorimetric assay (BCA Protein Assay, Pierce, Rockford, Illinois). The models of 17K(MXM) and 17K(M=M) were derived with the MOLSCRIPT program (Kraulis, 1991) from the structure of BS-RNase in the MXM form (PDM codename 1BSR), and from a model of M=M (Piccoli et al., 1992), respectively.

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