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Limited proteolysis of ribonuclease A with thermolysin in trifluoroethanol

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Abstract

We have examined the proteolysis of bovine pancreatic ribonuclease A (RNase) by thermolysin when dissolved in aqueous buffer, pH 7.0, in the presence of 50% (v/v) trifluoroethanol (TFE). Under these solvent conditions, RNase acquires a conformational state characterized by an enhanced content of secondary structure (helix) and reduced tertiary structure, as given by CD measurements. It was found that the TFE-resistant thermolysin, despite its broad substrate specificity, selectively cleaves the 124-residue chain of RNase in its TFE state (20–42 °C, 6–24 h) at peptide bond Asn 34–Leu 35, followed by a slower cleavage at peptide bond Thr 45–Phe 46. In the absence of TFE, native RNase is resistant to proteolysis by thermolysin. Two nicked RNase species, resulting from cleavages at one or two peptide bonds and thus constituted by two (1–34 and 35–124) (RNase Th1) or three (1–34, 35–45 and 46–124) (RNase Th2) fragments linked covalently by the four disulfide bonds of the protein, were isolated to homogeneity by chromatography and characterized. CD measurements provided evidence that RNase Th1 maintains the overall conformational features of the native protein, but shows a reduced thermal stability with respect to that of the intact species ($-\Delta T_m$ 16 °C); RNase Th2 instead is fully unfolded at room temperature. That the structure of RNase Th1 is closely similar to that of the intact protein was confirmed unambiguously by two-dimensional NMR measurements. Structural differences between the two protein species are located only at the level of the chain segment 30–41, i.e., at residues nearby the cleaved Asn 34–Leu 35 peptide bond. RNase Th1 retained about 20% of the catalytic activity of the native enzyme, whereas RNase Th2 was inactive. The 31–39 segment of the polypeptide chain in native RNase forms an exposed and highly flexible loop, whereas the 41–48 region forms a β-strand secondary structure containing active site residues. Thus, the conformational, stability, and functional properties of nicked RNase Th1 and Th2 are in line with the concept that proteins appear to tolerate extensive structural variations only at their flexible or loose parts exposed to solvent. We discuss the conformational features of RNase in its TFE-state that likely dictate the selective proteolysis phenomenon by thermolysin.

Keywords: CD; limited proteolysis; NMR; ribonuclease A; thermolysin; trifluoroethanol

Short-chain alcohols, such as methanol, ethanol, propanol, chloroethanol, and, in particular, trifluoroethanol, have been used frequently to induce α-helical conformations in otherwise randomly coiled or partially structured polypeptides (Toniolo et al., 1975; Nelson & Kallenbach, 1986; Lehrman et al., 1990; Segawa et al., 1991; Sönnichsen et al., 1992). It was shown that, on adding TFE to an aqueous solution of the S-peptide of bovine pancreatic ribonuclease A, this relatively small peptide acquires the helical conformation that it adopts in the native protein (Tamburro et al., 1968). Since then, TFE became the cosolvent of choice for inducing and stabilizing the helical secondary structure of peptides. The helix-inducing effect of TFE, however, is not occurring independently of the amino acid sequence of the polypeptide chain, but rather it seems that its main effect is that of increasing the helical population of those peptides and protein fragments that intrinsically show a high propensity to form helices (Lehrman et al., 1990; Segawa et al., 1991).

Weakly polar alcohols, including TFE, also have been shown to disrupt the native conformation of globular proteins, but the resulting denatured state was much different from the random-coil state observed in the presence of chemical denaturants such as urea

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Abbreviations: RNase, bovine pancreatic ribonuclease A (EC 3.1.27.5); RNase Th1, a folded, partially active derivative of RNase A with a chain fission at the peptide bond Asn 34–Leu 35 [RNase(1–34)(35–124)]; RNase Th2, a derivative of RNase A with chain fissions at peptide bonds Asn 34–Leu 35 and Thr 45–Phe 46 [RNase(1–34)(35–45)(46–124)]; TFE, 2,2,2-trifluoroethanol; [θ], mean residue ellipticity; TFA, trifluoroacetic acid; PTC, phenylthiocarbamoyl; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine; DTT, dithiothreitol; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; TSP, sodium 3-trimethylsilyl (2,2,3,3-$^2H_4$) propionate; T_m, the temperature at which a thermal transition is one-half complete; RP-HPLC, reverse-phase HPLC.

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or guanidine hydrochloride. Recent studies, based on CD and NMR measurements, have shown that the TFE-state of a protein, e.g., as obtained by dissolving the protein in 50% (v/v) aqueous TFE, is a stable, partially folded state with a high content of α-helical conformation, but lacking the specific tertiary interactions of the native protein (Buck et al., 1993, 1995; Fan et al., 1993; Alexandrescu et al., 1994; Shiraki et al., 1994). This TFE-state appears to be a noncompact, expanded conformational state characterized by an ensemble of fluctuating helices. However, the TFE-state does not result from a gross structural reorganization of the protein to another unrelated structure, because the large majority of amides protected from exchange in the TFE-state are also protected in the native state of the protein, thus implying a similar location of helical segments along the polypeptide chain in both native and TFE-state (Buck et al., 1993; Alexandrescu et al., 1994). One intriguing observation of these studies was that proteins in 50% (v/v) TFE acquire a higher content of helical secondary structure than the native species, implying that TFE also induces extra, non-native helices (Galat et al., 1985; Buck et al., 1993, 1995; Alexandrescu et al., 1994; Shiraki et al., 1994). Indeed, evidence has been provided that TFE, and other alcohols, can induce the α-helical conformation in globular proteins consisting mainly of β-sheets (Dufour & Haertlé, 1990; Fan et al., 1993; Liu et al., 1994; Shiraki et al., 1994). Overall, these various conformational properties of the TFE state can be related to those of the "molten globule" state of a protein, in which a polypeptide chain acquires a high content of secondary structure, and the side chains do not have fixed conformations (Kuwajima, 1989; Ptitsyn, 1992, 1995).

The precise physical mechanism of α-helix induction by TFE is not fully understood and is the subject of intense and recent investigations (Thomas & Dill, 1993; Jasanoff & Fersht, 1994; Shiraki et al., 1994; Cammers-Goodwin et al., 1996). Current views are that TFE favors the formation of the intramolecular hydrogen bonds of the α-helical conformation rather than hydrogen bonds to solvent. Even if helix induction and stabilization by TFE is the most frequently observed phenomenon, evidence for TFE-mediated stabilization of β-bends and β-hairpins in relatively short peptides has been provided (Blanco et al., 1994) and thus it may be proposed that aqueous TFE, in general, appears to strengthen the backbone–backbone hydrogen bonds.

The aim of the present work was to probe the conformational state of RNase when dissolved in aqueous TFE by using proteolytic enzymes as probes of protein structure and dynamics (Mihalyi, 1978; Neurath, 1980; Fontana et al., 1989, 1993; Price & Johnson, 1990; Signor et al., 1990; Wilson, 1991). This experimental approach relies on the fact that proteolysis of a polypeptide chain requires the binding of a chain segment of 6–8 amino acid residues of the polypeptide substrate at the active site cleft of the protease (Berger & Schechter, 1970). This is difficult to achieve with native, folded proteins, which are therefore usually quite resistant to proteolysis. Considering that native (N) proteins are in equilibrium with the unfolded, denatured (D) species, it is conceivable to suggest that protein degradation by proteolysis occurs via the unfolded species only, so that the N $\leftrightarrow$ D equilibrium actually dictates and control the rate of protein degradation to small peptides, if a protease of low substrate specificity is employed. Nevertheless, native globular proteins also can act as substrates for proteolysis, but, in this case, usually selective or preferential proteolysis at the level of very few peptide bonds is observed, leading to "nicked" protein species constituted by relatively large protein fragments associated in a stable and often functional complex (Mihalyi, 1978). A detailed survey of the structural characteristics of the sites of limited proteolysis in globular proteins of known three-dimensional structure enabled us to observe that peptide bond fissions are located at flexible loops of the protein and never at chain segments embedded in rigid secondary structure elements such as helices (Fontana et al., 1989, 1993; Polverino de Laureto et al., 1994a, 1994b). Thus, limited proteolysis of globular proteins is expected to occur at sites characterized by the motility required for an effective and stereospecific binding at the active site of the protease in order to form the idealized transition state of the hydrolytic reaction. Indeed, a clear-cut correlation exists between sites of limited proteolysis and sites of high segmental mobility (B-factor) determined crystallographically (Fontana et al., 1986). It is our belief that notions of exposure, accessibility, or protrusion (Novotny & Bruccoleri, 1987; Hubbard et al., 1991) are clearly not sufficient to explain the limited proteolysis phenomenon, because it is evident that, even in a small globular protein, there are many exposed sites (see also Hubbard et al., 1994).

For this study of limited proteolysis of a model protein in its TFE-state, the thermophilic enzyme thermolysin (Heinrikson, 1977; Holmes & Matthews, 1982) appeared to be a most suitable proteolytic probe, because of its noteworthy stability under relatively harsh solvent conditions, including aqueous organic solvents (Wayne & Fruton, 1983; Welinder, 1988; Kitaguchi & Klibanov, 1989) and broad substrate specificity (Heinrikson, 1977; Keil, 1982). Thus, it was anticipated that thermolysin would cleave the polypeptide chain of RNase in its TFE-state at sites characterized by the flexibility required for an efficient proteolysis and not by the specificity of the protease. Moreover, RNase was considered a convenient choice as a model protein because of the extensive literature regarding its structure, dynamics, folding, and function, and because limited proteolysis played an important role in the elucidation of its structure and mechanism of action (Richards & Wyckoff, 1971; Blackburn & Moore, 1982). The subtilisin-mediated cleavage of native RNase at the Ala 20–Ser 21 peptide bond leading to RNase S, the nicked protein species RNase[(1–20)(21–124)] (Richards & Vithayathil, 1959), can be considered the most classical and fruitful limited proteolysis experiment, which, over the years, enabled a number of additional studies of the RNase molecule that contributed a great deal to present day knowledge and understanding of protein structure and function.

Here we report the isolation and characterization of two nicked species of RNase with one (RNase Th1) and two (RNase Th2) peptide bonds cleaved along the disulfide-crosslinked, 124-residue chain of the protein. Structural properties of RNase dissolved in aqueous TFE that likely contribute to its specific nicking by thermolysin are discussed. Conformational and functional properties of the two nicked RNase molecules are interpreted, taking advantage of the known three-dimensional structure and dynamics of native RNase in the crystal state (Wyckoff et al., 1970; Wlodawer et al., 1982, 1988) or in solution (Rico et al., 1989; Santoro et al., 1993).[3]

Results

Proteolysis of RNase by thermolysin in the presence of TFE

Initial experiments of proteolysis of RNase by thermolysin in the presence of aqueous TFE were conducted by varying the alcohol

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concentration, the protease/RNase ratio, as well as the time and temperature of incubation. Samples were withdrawn periodically from the reaction mixture and proteolysis stopped by acidification. The pattern of protein fragmentation was monitored by SDS-PAGE under reducing conditions and utilizing the Tricine buffer system (Schägger & von Jagow, 1987) (Fig. 1). In the absence of TFE, RNase is resistant to proteolysis by thermolysin (3% by mass) when incubated in Tris buffer, pH 7.0, at 20–42 °C for several hours, because only the band of the intact protein (~14 kDa) was observed in the stained gel (Fig. 1). At higher temperatures (50–65 °C) and as the time of incubation increased (up to 24 h), the band corresponding to the intact protein disappeared gradually from the gel, whereas other bands of peptide/protein material were not seen in the gel. It is likely that the native-denatured conformational transition of RNase dictates the rate of protein degradation and that the unfolded protein is digested to small peptides not stained by the Coomassie dye of the SDS-PAGE system.

The results of the SDS-PAGE analysis (Fig. 1A) of the proteolytic mixture obtained by reacting RNase with thermolysin (4 h, 42 °C) in the presence of increasing concentrations of TFE (up to 50% by volume) show that RNase is cleaved if the alcohol concentration is >20%. The interesting observation is that few and rather large fragments are produced. In 30–50% TFE, three main protein fragments of approximately 10, 9, and 4 kDa are seen in the stained gel, besides traces of the intact protein (~14 kDa) (Fig. 1A). The SDS-PAGE analysis of the time-course proteolysis of RNase in 50% TFE at 20 °C is shown in Figure 1B. It is seen that the initial cleavage of the protein (after 4 h) leads to two main fragments of ~10 and ~4 kDa, implying that the protein is cleaved at a single peptide bond, considering that the sum of the molecular masses of the two fragments corresponds to that of the intact protein (~14 kDa). The gel of Figure 1B clearly shows that the ~10 kDa fragment is converted subsequently and slowly to a fragment of a lower mass, so that, after 30 h reaction, essentially a ~9-kDa and ~4-kDa fragment appear to be the end products of the reaction (note that small peptides are not stained in the gel). The pattern of proteolysis of RNase in 30% TFE was similar to that obtained in 50% TFE (see Fig. 1A). These last solvent conditions were used routinely for the preparative isolation of nicked RNase (see below), because this solvent mixture was used in previous studies of the TFE-induced structure of some proteins (Buck et al., 1993; Alexandrescu et al., 1994; Fontana et al., 1995; Polverino de Laureto et al., 1995a, 1995b).

In order to identify the thermolytic peptide bond fission(s) along the polypeptide chain of RNase, the peptide/protein bands of the SDS-PAGE gel were electroblotted (Matsudaira, 1987) onto a Pro-Blott membrane and then subjected to Edman degradation using the automatic sequencer (see Materials and methods). These preliminary N-terminal sequence data of the electroblotted fragments allowed us to suggest that the initial single nick of the chain occurs at peptide bond Asn 34–Leu 35, followed by an additional cleavage at Thr 45–Phe 46 (see below for more precise analytical data of the nicked RNase species isolated to homogeneity).

Isolation and characterization of nicked RNase

The results of SDS-PAGE analysis (above) were complemented by those obtained by ion-exchange and RP-HPLC. In particular, column chromatography was employed for the purification of nicked RNase species in sufficient quantity for subsequent analysis. It was clear from the SDS-PAGE analysis (Fig. 1B) that a proteolytic mixture leading mostly to the hydrolysis of a single peptide bond (see above) was that resulting from a reaction conducted in 50% TFE at 20 °C for 4 h (Fig. 1B). In practice, we have found that proteolysis at 42 °C for 30 min leads to a quite similar proteolytic mixture. A sample of this mixture was applied to a Mono-S column, which was eluted with a salt gradient (Fig. 2A). The peptide/protein material of the major peak of the chromatogram (named Th1, Fig. 2A) gave two bands in the SDS-PAGE gel (reducing conditions) of ~10 and ~4 kDa, respectively.

In order to produce a nicked RNase with more than one peptide bond hydrolyzed (see above), proteolysis was conducted in 50% TFE for 6 h at 42 °C. This sample was applied to a Vydac C_4 column eluted with a gradient of acetonitrile. The chromatogram of Figure 2B shows that a major peak of protein material is eluted from the column at ~37% acetonitrile. This protein sample (named RNase Th2) was shown by SDS-PAGE (reducing conditions) to be constituted by two fragments of molecular masses of ~9 kDa and ~4 kDa.

The disulfide-crosslinked protein material RNase Th1 and RNase Th2, isolated by ion-exchange and RP-HPLC, respectively, was reduced with DTT and subsequently S-carboxamidomethylated (see Materials and methods). From these reaction mixtures, the protein

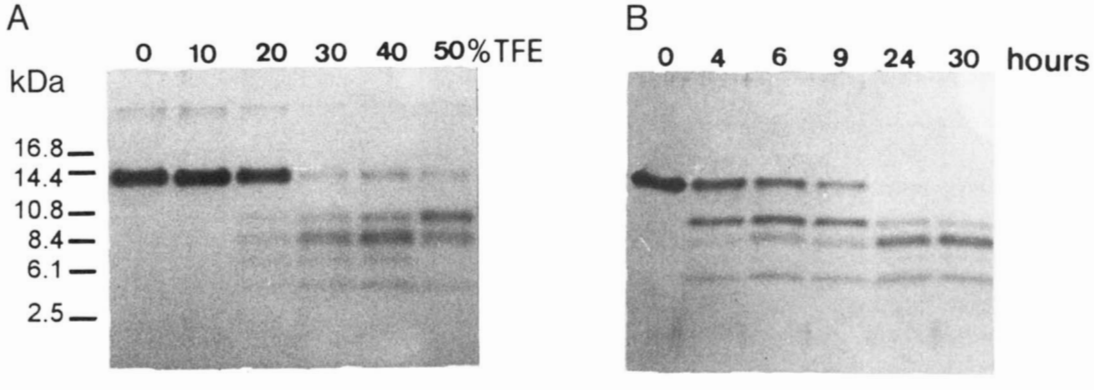

Fig. 1. SDS-PAGE of proteolytic mixture of RNase with thermolysin. **A:** Reaction proceeded for 4 h at 42 °C, in 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.0, at an enzyme to substrate ratio of 3% (by weight) in the presence of TFE from 0 to 50% (by volume). Molecular weight markers are shown on the left in kDa. **B:** Time-course of the proteolysis of RNase with thermolysin in Tris buffer containing 50% TFE at 20 °C.

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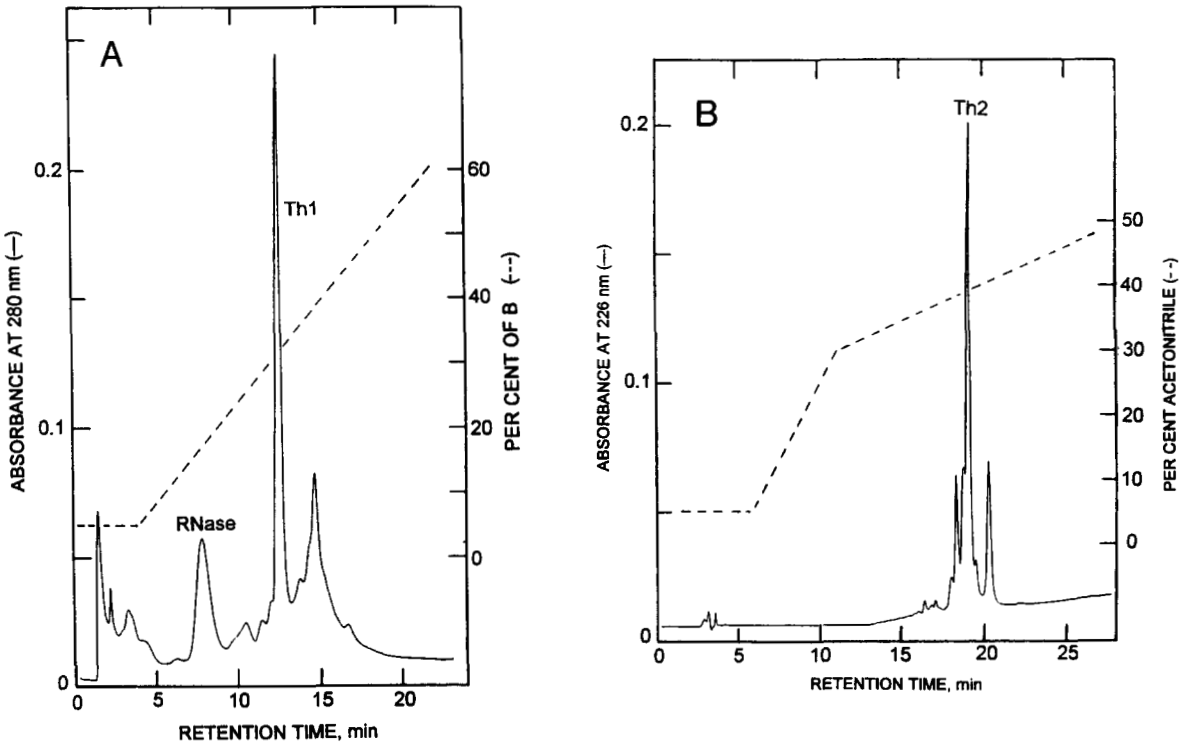

Fig. 2. Chromatographic analysis of the proteolytic mixture of RNase with thermolysin. **A:** Ion-exchange HPLC of RNase reacted with thermolysin in 50% TFE. Reaction proceeded for 30 min at 42 °C using an enzyme/substrate ratio of 3% (by weight) in 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$ and 50% TFE. The separation was performed on a Mono-S HR 5/5 column (Pharmacia-LKB), equilibrated with 50 mM KH_2PO_4 buffer, pH 7.0, and eluted with a gradient (B) of the same buffer containing 0.8 M KCl. **B:** RP-HPLC of RNase reacted with thermolysin in 50% TFE. Proteolysis was conducted under the same conditions given in A and allowed to proceed for 6 h at 42 °C. The separation was performed on a Vydac C4 column eluted at a flow rate of 0.6 mL/min with a gradient of acetonitrile containing 0.05% TFA (by volume).

fragments constituting RNase Th1 and RNase Th2 were isolated by RP-HPLC (see Fig. S.2, Electronic Appendix) and subjected to further analysis (amino acid composition after acid hydrolysis, N-terminal sequencing, and determination of the molecular mass by mass spectrometry). The analytical data of the fragments, as well as those of the parent disulfide-crosslinked protein species (see Table S.1, Electronic Appendix), when compared with the known amino acid sequence of RNase (Smyth et al., 1963; see Fig. S.1, Electronic Appendix), allowed us to establish unambiguously the chemical identity of the nicked protein species. RNase Th1 is a nicked protein with the single Asn 34–Leu 35 peptide bond hydrolyzed, whereas RNase Th2 is with both Asn 34–Leu 35 and Thr 45–Phe 46 hydrolyzed. RNase Th1 is therefore constituted by two fragments, 1–34 and 35–124, and RNase Th2 by three fragments, 1–34, 35–45, and 46–124. It should be noted that the fragments in both RNase Th1 and Th2 are linked covalently by disulfide bonds, because the eight cysteine residues of the RNase molecule form four disulfide bridges between residues 26 and 84, 40 and 95, 58 and 110, and 65 and 72 (Smyth et al., 1963).

Because the aim of the present work was also to characterize the structural and functional properties of the nicked protein species, samples of purified RNase Th1 and RNase Th2 were further analyzed by RP-HPLC and by capillary electrophoresis. The results of these analyses demonstrated that indeed the isolated protein samples were highly homogeneous (Fig. 3). Moreover, gel filtration experiments also allowed us to establish the purity of nicked proteins (not shown). Of note, RNase Th1 eluted from a Superose-12 column at the same position as that of the intact protein, whereas RNase Th2 eluted somewhat earlier, signifying a higher hydrodynamic volume of RNase Th2 with respect to that of intact and RNase Th1 and therefore a more expanded protein species (Corbett & Roche, 1984).

CD measurements

Intact RNase in TFE

The effect of increasing concentrations of TFE on the conformation of intact RNase was investigated by far-UV (Fig. 4A) and near-UV (Fig. 4B) CD spectroscopy. If dissolved in 30% aqueous TFE at pH 7.0, RNase acquires a slightly enhanced content of α-helix with respect to that of the protein in buffer alone. In fact, the intensity of the negative ellipticity in the 200–250-nm region of RNase in aqueous TFE is greater than that of the protein in buffer only, and shows characteristics typical of helical polypeptides with minima of ellipticity near 208 and 222 nm (Greenfield & Fasman, 1969; Johnson, 1990). The enhanced content of helicity is much more evident if the protein is dissolved in 50% TFE (Fig. 4A, curve 3). A quantitative analysis of the far-UV CD spectra in terms of percentages of secondary structure (Chen et al., 1972) allowed us to estimate that RNase acquires a conformational state characterized by 33% and 45% helicity in 30% and 50% TFE, respectively. These figures should be compared with the 30% helical content of native RNase, derived from the crystal structure of the protein.

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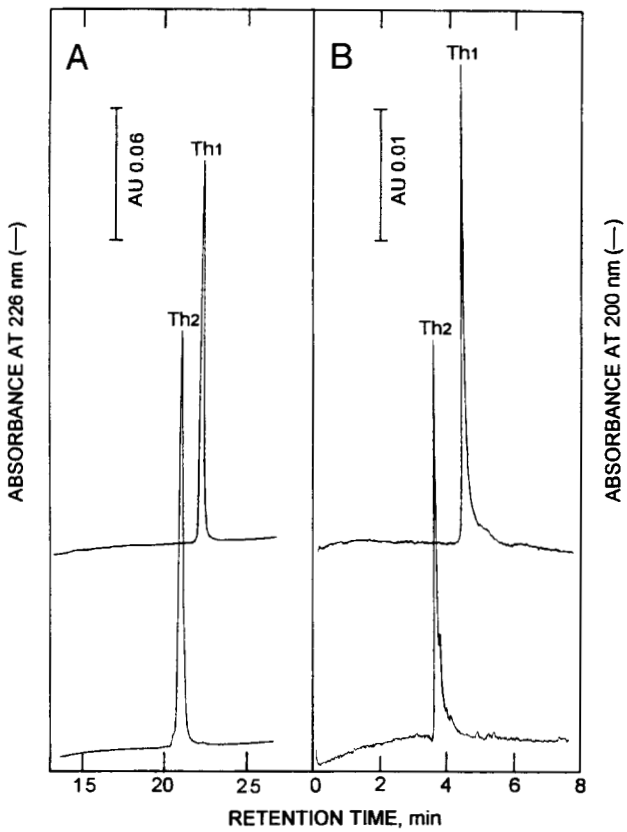

Fig. 3. RP-HPLC and capillary zone electrophoresis of nicked RNase Th1 and RNase Th2. **A:** HPLC analysis was performed on a Vydac C4 column eluted at a flow rate of 0.6 mL/min with a linear gradient of acetonitrile containing 0.05% TFA (by volume) from 5 to 22% in 5 min and from 22 to 50% in 17 min. Effluent was monitored at 226 nm. **B:** Capillary electrophoresis was performed in Na-phosphate buffer, pH 2.5, on a coated capillary (20 cm × 25 μm) at 12 kV. Effluent was monitored at 200 nm.

Near-UV CD spectra show that the addition of TFE to an aqueous solution of RNase leads to a partial disruption of its tertiary structure. As shown in Figure 4B, the CD spectrum in the 240–310-nm region of RNase dissolved in 30% TFE still maintains the overall features of the spectrum of the native species in buffer only, even if the intensity of the CD signal is somewhat reduced (15%). In contrast, the CD spectrum of RNase in the presence of 50% TFE (Fig. 4B, curve 3) shows a marked reduction of negative ellipticity in the 260–290-nm region with respect to that of the protein in buffer only, indicating that the TFE-state of RNase in 50% TFE is characterized by a severe reduction of tertiary interactions.

Nicked RNase

The structural consequence of the peptide bond fission(s) in the RNase molecule were investigated by far-UV (Fig. 5A) and near-UV (Fig. 5B) CD spectroscopy. Between 200 and 250 nm, the CD spectrum of RNase Th1 is very similar to that of the intact protein, indicating that the secondary structure of the protein is unchanged after fission of the peptide bond Asn 34–Leu 35. On the other hand, the CD spectrum of RNase Th2, with two peptide bonds broken, is largely changed with respect to that of the intact protein and displays features most typical of a random-coil polypeptide (Greenfield & Fasman, 1969; Johnson, 1990).

RNase Th1 shows negative ellipticity values in the 260–290-nm region, somewhat lower (10%) than those of the intact protein (Fig. 5B). The overall shape and fine structure of the near-UV CD spectrum, measured at 25 °C, is, however, similar for both protein species. When the CD spectrum of RNase Th1 was instead measured at 5 °C (not shown), the spectrum in the 250–300-nm region was essentially identical for both intact and nicked protein. Because the near-UV CD spectrum of a protein is due to the contributions of aromatic residues and disulfide bonds (Strickland, 1974), near-UV CD spectroscopy indicates a close similarity of tertiary structure between RNase Th1 and the intact native protein. On the other hand, the strong reduction of the CD signal in the 250–300-nm region observed with RNase Th2 can be taken as a clear indication that the tertiary structure is largely disrupted if the polypeptide chain of RNase has suffered fissions at two peptide bonds.

NMR spectroscopy

Intact RNase in TFE

TOCSY and NOESY spectra were obtained for RNase A in the mixed-solvent 30% TFE. They correspond essentially to a superposition of the spectra of a native-like protein and a TFE-denatured form with relative populations of 30% and 70%, respectively. In the latter form, the high dispersion in chemical shifts, typical of packed globular proteins, is absent. For the native-like form, chemical shifts (δ) of CH protons are all within 0.10 ppm of those of the intact protein. Shift differences for NH amide protons are larger, amounting in one case to 0.40 ppm. Most of the observed shifts are toward lower δ-values, reflecting differences in solvation with respect to the aqueous solution and, more particularly, the much weaker basic properties of TFE (Llinás & Klein, 1975). The NOE patterns for every proton are similar to those observed for the intact protein, implying that a native-like form of the RNase persists in the mixed solvent. Because the signals of the native-like species and those of the TFE-denatured form, which are mainly evident in the δ 7.2–6.8-ppm region corresponding to random-coil tyrosine aromatic protons, do not show appreciable line broadening, it can be proposed that the two forms are in slow exchange with respect to the NMR time scale. No information could be drawn for the TFE-denatured form because of the poor chemical shift dispersion in the spectrum, which makes overlapping very severe.

Nicked RNase

The ^{1}H NMR spectral assignment of nicked RNase Th1 was achieved by means of the standard sequential assignment strategy (Wüthrich, 1986). The process was largely facilitated by the previous assignment for the intact protein (Rico et al., 1989; Robertson et al., 1989). In general, the location of most of the signals, i.e., the fingerprint region of the TOCSY spectrum, was followed easily because of their proximity to the corresponding signals of the intact protein. Only the assignment of residues 30–40 had to be made on a completely independent basis. Fairly intense amide–amide sequential connections were observed in the NOESY spectra for residues in the segment 29–34, with the exception of the one between residues 32 and 33, which must be occluded by diagonal signals. Sequential CH_i-$NH_{(i+1)}$ cross-correlations were observed for all pairs in segment 29–34, as well as those corresponding to segment 35–41. Sequential amide–amide cross-correlations were also observed for residues in segment 37–40. Table 1 lists the chemical shifts of the proton resonances of RNase Th1 corresponding to residues in the cleaved region (Met 29–Pro 42), together with those of the intact protein. The differences between the chem-

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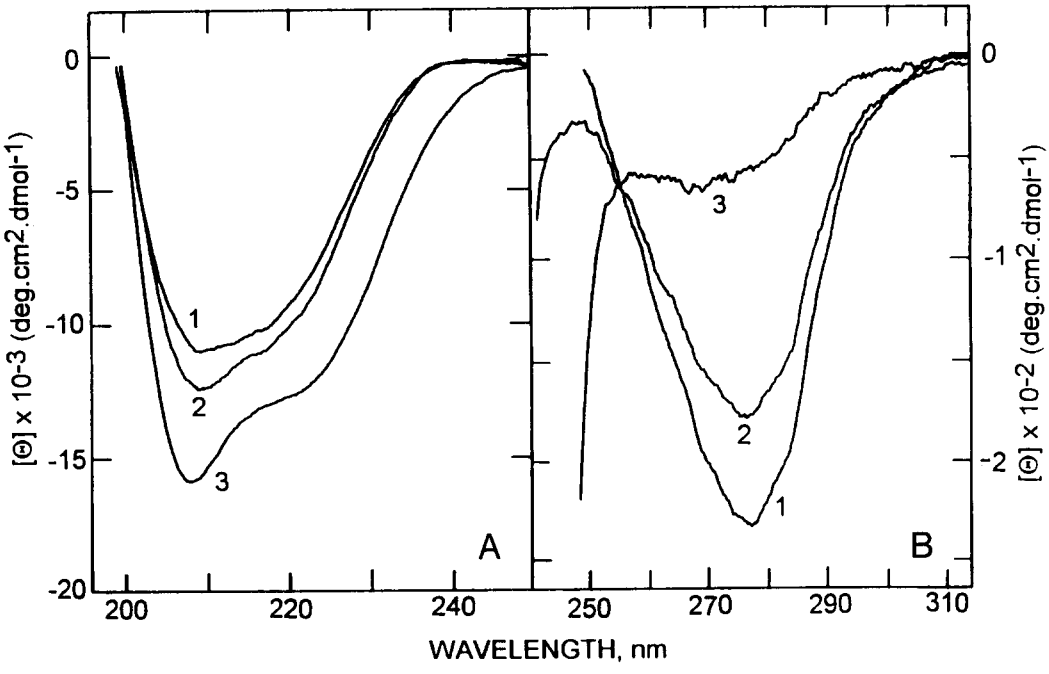

Fig. 4. Effect of TFE on the (**A**) far-UV and (**B**) near-UV CD spectra of RNase. Protein was dissolved in 50 mM Tris-HCl, pH 7.0, in the absence of TFE (curve 1) and in the presence of 30% TFE (curve 2) and 50% TFE (curve 3). Protein concentration was 0.1 and 0.5 mg/mL for CD measurements conducted in the far-UV and near-UV region, respectively.

ical shifts (δ) of CH and NH resonances between RNase Th1 and the intact protein were plotted versus the sequence number. These chemical shifts (δ) depend on both the nature of the amino acid and on the electromagnetic environment of the peptide bond (Wüthrich, 1986; Wishart et al., 1991).

As shown in Figure 6, the largest differences in chemical shifts are largely confined at residues located nearby the cleaved peptide bond Asn 34–Leu 35. Differences as large as 1.7 ppm (Lys 41-NH) and some others larger than 0.5 ppm have been detected for protons in residues in that region. These differences in chemical shift have their origin in (1) the newly generated $-COO^-$ and NH_3^+ groups at residues 34 and 35; (2) the conformational change undergone by the residues located near the hydrolyzed peptide bond, most probably toward more flexible conformations; (3) the effect of electric and magnetic anisotropy of peptide groups on the corresponding protons, which will be nonvanishing in the intact protein; and (4) ring current effects arising from the nearby aromatic rings of Tyr 92 and Tyr 97 and even those of His 12, Tyr 25, and Phe 46. Moreover, differences in chemical shifts can be observed in regions far in the sequence but proximate in the space, like those in the chain segment Lys 7–Asp 14 and Arg 85–Cys 95. Long-range tertiary effects of anisotropic groups on protons in the intact protein are probably the main origin of the observed differences in chemical shifts. Chemical shifts in all other regions of the protein are nearly identical, perhaps with the exception of some resonances shifted by small differences in pH (Phe 120-NH). Also, the NOE patterns observed for all these protons correspond very closely with those shown by the analogous protons in the intact protein. Altogether, these NMR results confirm unambiguously that RNase Th1 has a cleaved 34–35 peptide bond and also that the structure of this nicked protein is closely similar to that of the intact form (Santoro et al., 1993).

Thermal denaturation of RNase

Fission of the Asn 34–Leu 35 peptide bond destabilizes RNase markedly. The thermal unfolding transitions of both nicked RNase Th1 and intact protein were measured at pH 7.0 by monitoring the temperature dependence of the CD signal at 220 nm. The midpoint of the thermal transition of RNase Th1 is reduced by 16 °C with respect to that of the intact protein (Fig. 7).

Enzymatic activity

The breaking of the peptide bond Asn 34–Leu 35 in RNase leads to a reduction of the enzymatic activity. With cytidine 2′,3′-phosphate as substrate, the relative activity at 25 °C of RNase Th1 is decreased to ~20% with respect to that of the intact enzyme. On the other hand, the RNase Th2 species, with peptide bonds Asn 34–Leu 35 and Thr 45–Phe 46 hydrolyzed, was inactive.

Discussion

Limited proteolysis

The results presented in this paper show that thermolysin cleaves RNase in aqueous TFE at a very restricted region of its 124-residue polypeptide chain. The peptide bond Asn 34–Leu 35 is the site of selective initial cleavage, followed by an additional, but slower, cut at Thr 45–Phe 46. This selective hydrolysis is quite striking, if one considers that thermolysin shows a broad substrate specificity with only some preferences for hydrophobic and bulky amino acid residues (Ile, Leu, Phe) (Heinrikson, 1977; Keil, 1982). Therefore, there are plenty of peptide bonds in the RNase polypeptide chain (see Fig. S.1, Electronic Appendix) that can be cleaved by thermolysin and, because this is not occurring, we must propose that

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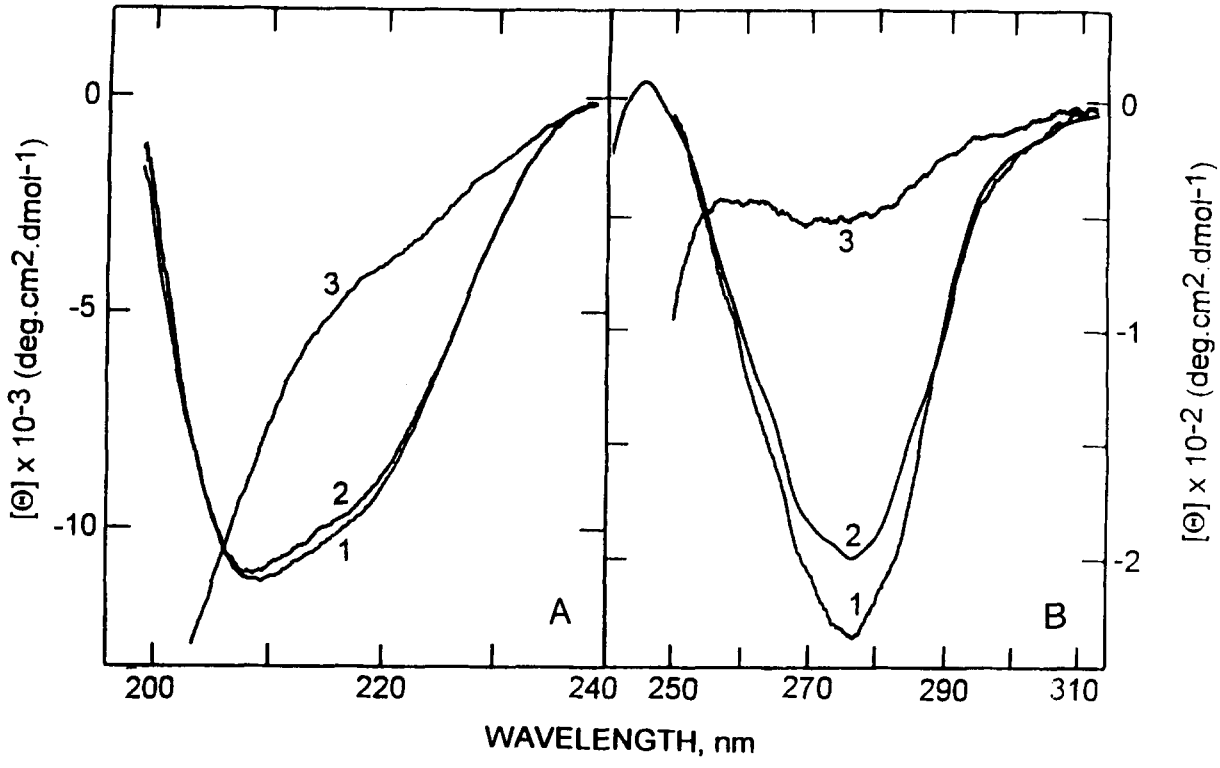

Fig. 5. Far-UV and near-UV CD spectra of RNase and its nicked species. Spectra were taken at 20 °C in 50 mM Tris-HCl buffer, pH 7.0. 1, RNase; 2, RNase Th1; 3, RNase Th2.

these peptide bonds in the RNase molecule dissolved in 30–50% TFE are embedded in a rigid protein structure and thus not amenable to proteolytic digestion. Accepting our view that segmental mobility is the most critical parameter in dictating selective proteolysis of a globular protein (Fontana et al., 1986, 1993), it can be proposed that, with RNase in aqueous TFE, only the chain segment encompassing the site(s) of cleavage is flexible enough to bind easily and properly at the active site of thermolysin in order to facilitate peptide bond fission (Hubbard et al., 1994) (see also Polverino de Laureto et al., 1995b, for additional comments on the mechanism of selective proteolysis of proteins by thermolysin in aqueous TFE).

The conformational and dynamic features of RNase in relatively high concentrations of TFE are not yet known in such detail as for other proteins, e.g., lysozyme (Buck et al., 1993, 1995), α-lactalbumin (Alexandrescu et al., 1994), and tendamistat (Schönbrunner et al., 1996). Nevertheless, the results of CD and NMR measurements of RNase dissolved in aqueous TFE allow us to propose a possible model of the TFE state of RNase. First of all, CD data show clearly that aqueous TFE enhances the helical content of the native protein (far-UV), with concomitant loss of the well-defined tertiary structure (near-UV) of the native protein (see Fig. 4). In 30% TFE, RNase appears to maintain the gross conformational features of the native protein, as given by both far-UV and near-UV CD data (Fig. 4), whereas, in 50% TFE, the helical content of the protein is enhanced and the tertiary structure is disrupted significantly. Preliminary NMR data of RNase in 30% TFE provided evidence of the existence of two conformational states of the protein in slow exchange, the minor one (~30%) being similar to that of the native one. The major form (~70%) has lost the NMR dispersion signals and it appears to be a TFE-denatured state. Even if additional NMR experiments are required to shed light on the molecular aspects of the TFE-induced conformational transition of RNase, at present we can speculate that RNase in the presence of relatively high concentrations of TFE (e.g., 30–50%) can attain an expanded globular form maintaining some structural features of the native protein, in equilibrium with an extended and highly helical form (ensemble of fluctuating helices), which can be considered a TFE-denatured state. The expanded, native-like species of RNase would be largely populated in 30–50% TFE, the solvent conditions utilized in the present work for the limited proteolysis of RNase. This state would be sufficiently flexible at the site of proteolytic cleavage (see also below) and rigid enough in other parts of the protein molecule to prevent extensive hydrolysis. It could well be that the irregular chain region near the Asn 34–Leu 35 peptide bond in native RNase (see Fig. 8), even in aqueous TFE, does not adopt a persistent secondary structure. Moreover, we advance the hypothesis that the highly helical form attained by RNase in aqueous TFE, is not, or at least comparatively very slowly, attacked by thermolysin, if one accepts our view that limited proteolysis does not occur at helical chain segments (Fontana et al., 1986, 1993; Polverino de Laureto et al., 1994a, 1994b).

Proteolytic enzymes have been used in the past to probe the partial unfolding of RNase during thermal denaturation (Rupley & Scheraga, 1963; Ooi & Scheraga, 1964; Winchester et al., 1970) or in the presence of low concentrations of guanidine hydrochloride (Yang & Tsou, 1995). Briefly, the sites of initial cleavages observed in these previous studies were also located at the same or near the chain segment shown to suffer proteolysis in the present

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Table 1. *Chemical shifts (δ, ppm from TSP) of proton resonances of RNase Th1 and RNase A (italics) corresponding to residues in the cleaved region*[a]

Residue	NH	H_α	H_β-$H_{\beta'}$	H_γ-$H_{\gamma'}$	H_δ-$H_{\delta'}$	H_ϵ-$H_{\epsilon'}$	H_η-$H_{\eta'}$
Met 29	8.30	4.08	0.97, 0.73	1.40, 1.05		1.32	
	8.50	*4.14*	*0.94, 0.85*	*1.37, 1.30*		*1.23* (Me)	
Met 30	8.49	4.50	1.92, 1.70	2.35, 0.91		1.60	
	8.68	*4.26*	*1.73, 1.40*	*2.44, 0.58*		*1.50* (Me)	
Lys 31	7.02	4.30	1.95, 1.95	1.41	1.60	3.02	
	6.57	*4.36*	*1.38, 1.89*	*1.43*	*1.71*	*3.01*	7.50
Ser 32	7.68	4.51	4.05, 3.90				
	8.60	*4.19*	*3.98, 3.91*				
Arg 33	7.73	4.27	1.60, 1.29	1.45	2.99, 2.91	7.06	
	7.80	*4.44*	*1.83, 2.27*	*1.85, 1.78*	*3.42, 3.12*	*7.30*	
Asn 34	8.20	4.30	2.48				
	7.95	*4.82*	*3.17, 2.95*		*7.37, 6.77*		
Leu 35		4.14	1.76	1.55	1.10, 0.96		
	8.12	*4.63*	*2.00, 1.60*	*1.55*	*0.89, 0.77*		
Thr 36	8.63	4.40	4.17	1.24			
	7.64	*5.40*	*4.84*	*1.20*			
Lys 37	8.41	4.26	1.75	1.40	1.73	2.95	7.51
	7.01	*4.20*	*1.80*	*1.46, 1.39*		*3.04*	
Asp 38	8.60	4.66	2.99, 2.84				
	8.81	*4.39*	*2.77, 2.61*				
Arg 39	7.74	4.48	1.70	1.76, 1.67	3.37, 3.12	7.36	
	7.72	*3.64*	*1.78, 1.52*	*1.39, 1.10*	*3.05*	*7.20*	
Cys 40	7.43	4.65	2.95, 2.78				
	9.00	*4.78*	*2.79, 2.94*				
Lys 41	9.01	4.55	1.80	1.52	1.70, 1.60	2.98, 2.84	
	7.33	*4.48*	*2.00, 1.63*	*1.64, 1.51*	*1.83, 1.47*	*3.34, 3.19*	
Pro 42	—	4.54	2.50, 2.13	2.23, 2.13	4.16, 4.00		
		4.53	*2.48, 2.13*	*2.25, 2.15*	*4.09, 3.92*		

[a]Measurements were performed at 35 °C, pH 4.0.

study. For example, at 60 °C, the initial cleavages of RNase by trypsin (Ooi & Scheraga, 1964) occur at Lys 31–Ser 32, Arg 33–Asn 34, and Lys 37–Asp 38, whereas chymotrypsin (Rupley & Scheraga, 1963) splits rapidly Tyr 25–Lys 26, followed by Leu 35–Thr 36 and Phe 46–Val 47. More recently, Arnold et al. (1996) reinvestigated the use of proteolytic probes for the thermal unfolding of RNase utilizing several proteases. Interestingly, these authors also used thermolysin in their study and concluded that initial cleavages on heating occur at the same peptide bonds here found to be cleaved in RNase dissolved in aqueous TFE. These previous results allow us to propose that both TFE and heat induce a relaxed state of the RNase molecule, characterized by a highly flexible 30–46 chain segment favoring selective proteolysis. After the initial cut at Asn 34–Leu 35, the resulting RNase Th1 molecule is strongly destabilized (see below) and suffers an additional cut at the Thr 45–Phe 46 peptide bond.

Structure–stability–function of nicked RNase

Although RNase Th2 was inactive toward cytidine 2′,3′-phosphate, RNase Th1 retained enzymatic activity, even if lower (~20%) than that of the intact protein. Both CD and NMR measurements were used by us to correlate the enzymological properties of the nicked proteins with their conformational features. Far-UV and near-UV CD data indicate that RNase Th2 is a largely unfolded molecule, thus explaining the lack of activity of this nicked species. RNase Th1 instead possesses the same secondary structure and a slightly perturbed tertiary structure with respect to those of the intact protein, as given by CD measurements (see Fig. 5). Of interest, the CD data of RNase Th1 closely parallel those of RNase S, the enzymatically active nicked protein obtained by limited proteolysis of RNase with subtilisin (cleavage at the Ala 20–Ser 21 peptide bond) (Strickland, 1974).

A more detailed structural analysis of the RNase Th1 molecule was conducted by 2D-NMR measurements. The differences in the chemical shifts (δ) of the CH and NH resonances between RNase Th1 and the native protein were measured as a function of the amino acid sequence. The figures for δ depend on both the nature of the amino acid and on the electromagnetic environment of the peptide bond (Wüthrich, 1986; Wishart et al., 1991). The measured differences in δ between the nicked and intact protein are largely confined at the chain segment where proteolytic cleavage oc-

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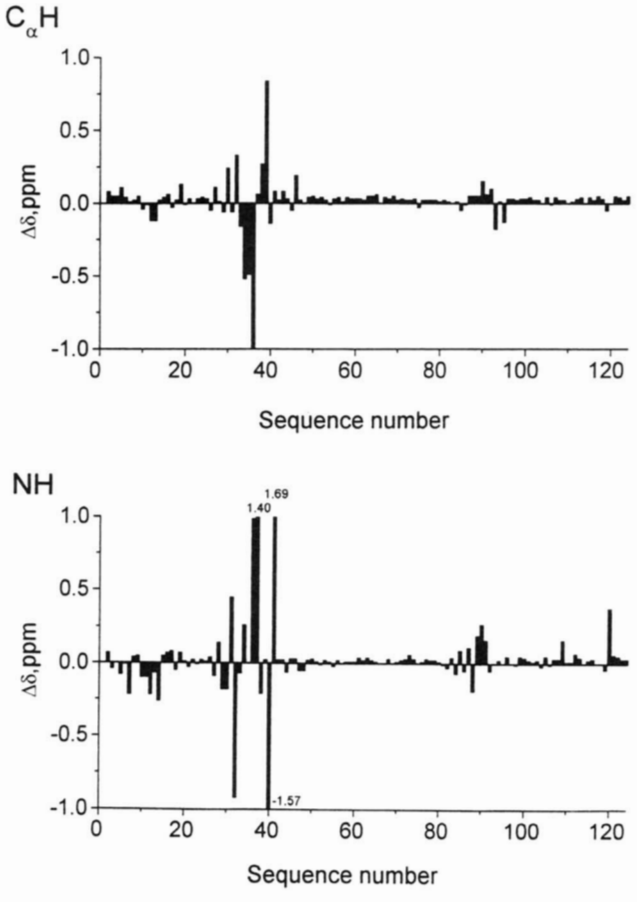

Fig. 6. Plots of the differences in the chemical shifts (δ) of the CH (top) and NH (bottom) resonances between RNase Th1 and the native protein versus amino acid sequence.

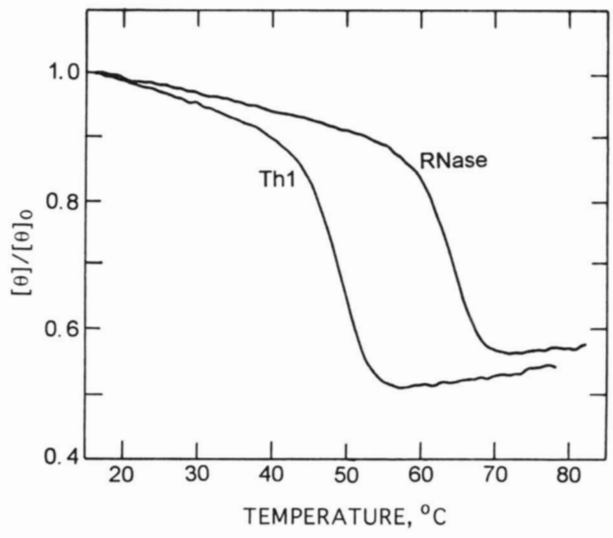

Fig. 7. Temperature dependence of the mean residue ellipticity, $[\theta]$, at 220 nm of nicked RNase Th1 and intact RNase. Melting profile of the proteins was measured in 50 mM Tris-HCl buffer, pH 7.0 (see Materials and methods). $[\theta]_0$ is the ellipticity measured at 15 °C.

curred. Therefore, 2D-NMR spectra indicate that, apart from the expected local changes around the site of peptide bond fission, the backbone conformation and the tertiary interactions of RNase Th1 are much similar to those of the intact protein.

Current understanding of protein structure–stability relationships, as well as the structural data available for intact RNase, allow us to explain why the fission of the Asn 34–Leu 35 peptide bond does not lead to major alteration of the structure of the protein (see above). First of all, the results of numerous experiments of site-directed mutagenesis conducted in recent years on a number of (model) proteins have indicated clearly that mutations can be introduced at surface sites, and in particular at chain loops, without major effects on protein structure and stability, whereas mutations of the rigid core of the protein, or those disrupting secondary structure elements, are much more detrimental (Alber, 1989; Fontana, 1991; Matthews, 1996). The site of fission in RNase Th1 (Asn 34–Leu 35, see Fig. 8) is located at a flexible loop displaying the largest dispersion values of the backbone angles of the RNase molecule, as given by NMR spectroscopy (Santoro et al., 1993). Even the refined X-ray structure of RNase revealed that "Asn 34 is located in a surface region with uncertain electron density and its conformation is still unknown," thus indicating a static/dynamic disorder of this site in the intact protein (Wlodawer et al., 1982). Indirect evidence of the flexibility of chain segment 31–39, forming a loop in the native RNase, was obtained by monitoring the refolding process of RNase by using trypsin as a proteolytic probe of protein structure. It was found that this segment is not protected significantly from tryptic attack during the folding of the protein (Lang & Schmid, 1986), implying that this site maintains some chain flexibility even if a large portion of the RNase molecule is already refolded to a native-like form. Finally, hydrogen exchange measurements by NMR of RNase under mildly denaturing conditions revealed that chain segment 30–36 exchanges protons at a much faster rate than other sites of the protein, and that the rate was too fast to be measured (Kiefhaber & Baldwin, 1995). All these structural data therefore indicate that the site

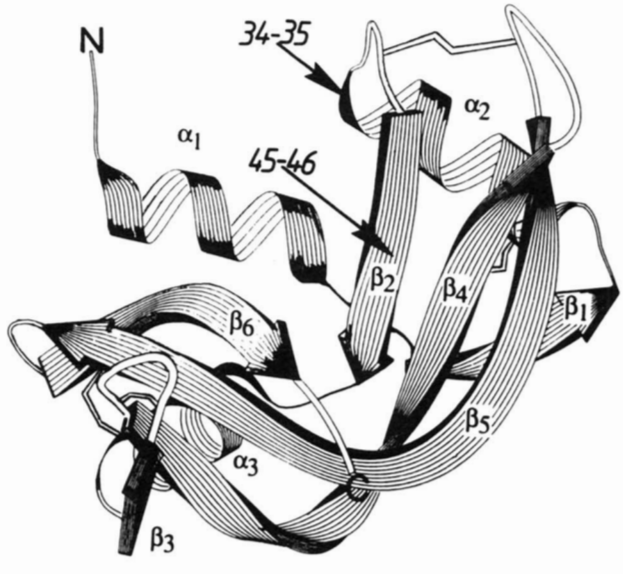

Fig. 8. Ribbon diagram of the three-dimensional structure of RNase. The α-helices (α_1–α_3) and β-sheets (β_1–β_6) are depicted in this diagram (adapted from Kolbanovskaya et al., 1992). Arrows point toward the sites of proteolysis.

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of initial thermolytic cleavage is exposed and flexible in the intact protein and thus cleavage of the Asn 34–Leu 35 peptide bond does not lead to disruption of a rigid structural moiety. On the other hand, in RNase Th2, the additional cleavage of the Thr 45–Phe 46 peptide bond is very disadvantageous thermodynamically because, after cleavage, the β-sheet encompassing this peptide bond (see Fig. 8) is likely destroyed and thus all hydrogen bonds cooperatively stabilizing this element of secondary structure are broken.

RNase Th1 was found less stable to heat than the intact protein ($-\Delta T_m$ 16°C), whereas RNase Th2 was fully unfolded at room temperature. The peptide bond hydrolysis is expected to destabilize a protein for purely entropic reasons, analogous to the reductive cleavage of a disulfide bond in a protein (Johnson et al., 1978; Pace et al., 1988). In fact, the destabilization due to fission of a covalent bond is related to the entropy gain in the denatured state, even if enthalpy and heat capacity effects may be included (see Krokoszynska & Otlewski, 1996, for a recent discussion). Of interest, the same $-\Delta T_m$ of 16 °C was determined previously for the difference in heat stability between nicked RNase S and intact RNase (Takahashi et al., 1969).

Concluding remarks

The results of this study show that proteolysis of RNase in aqueous TFE occurs very selectively, enabling the isolation of nicked protein species of structural and functional interest. Because RNase is one of the most studied protein molecules and currently is a prototype model protein utilized by many investigators to study structure–stability–folding aspects of proteins, likely the procedure described here can be utilized to produce interesting nicked proteins within the RNase superfamily (Beintema, 1987; Eftink & Biltonen, 1987). This study and our previous ones (Fontana et al., 1995; Polverino de Laureto et al., 1995a, 1995b) show that proteolysis of proteins in their TFE-state by the TFE-resistant thermolysin is very selective and that it can be used as a procedure to produce nicked proteins or, in general, rather large protein fragments for protein chemistry and/or biophysical studies. Finally, the results reported here provide some insights into the partly folded TFE-state of proteins utilizing a proteolytic probe, and complement those obtained by other investigators using spectroscopic techniques (Buck et al., 1993, 1995; Alexandrescu et al., 1994; Shiraki et al., 1994; Schönbrunner et al., 1996). This study represents a continuation of our efforts to demonstrate that proteolytic enzymes can be used as reliable probes of protein structure and dynamics (Vita et al., 1985; Fontana et al., 1989, 1993; Signor et al., 1990; Polverino de Laureto et al., 1994a, 1994b, 1995a, 1995b; Vindigni et al., 1994).

Materials and methods

Materials

Bovine pancreatic RNase (type XII-A) and thermolysin from *Bacillus thermoproteolyticus* were purchased from Sigma (St. Louis, Missouri) and used without further purification. The reagents used for N-terminal sequence analysis were from Applied Biosystems (Foster City, California) and those for amino acid analysis were from Waters-Millipore (Milford, Massachusetts). All other chemicals were of analytical reagent grade and were obtained from Sigma or Fluka (Buchs, Switzerland).

Proteolysis of RNase with thermolysin

Limited proteolysis of RNase with thermolysin was performed in 50 mM Tris-HCl buffer, pH 7.0, containing 5 mM $CaCl_2$, in the presence of increasing concentrations of TFE, using an enzyme to substrate ratio of 3:100 (by weight) for 45 min or 6 h at 20–22, 42, or 50 °C. The products of proteolytic cleavage were purified by ion-exchange chromatography on a Mono-S HR 5/5 column (Pharmacia-LKB, Uppsala, Sweden), eluted with a linear gradient of 50 mM KH_2PO_4 buffer, pH 7.0, containing from 0 to 0.8 M NaCl (0–60% in 19 min), or by RP-HPLC on a Vydac C_4 column (The Separations Group, Hesperia, California) using a linear gradient of acetonitrile containing 0.05% TFA (by volume) from 5 to 22% in 5 min and from 22 to 50% in 17 min. The elution conditions were at a flow rate of 0.6 mL/min and the effluent was monitored at 226 nm.

Reduction and S-alkylation of nicked forms of RNase

Reduction of disulfide bonds of nicked forms of RNase was performed with DTT in 0.5 M Tris-HCl buffer, pH 8.0, containing 6 M guanidine hydrochloride and 2 mM EDTA. The reaction mixture was kept at 37 °C for 3 h under nitrogen atmosphere and then 50 equivalents of iodoacetamide were added to the reaction mixture. After 1 h in the dark, excess DTT and reaction byproducts were removed by RP-HPLC on a Vydac C_4 column using the same acetonitrile/water gradient employed for the purification of RNase Th1 and Th2.

Electrophoresis

SDS-PAGE was performed using the Tricine buffer system described by Schägger and von Jagow (1987). Capillary zone electrophoresis was performed on a Bio-Rad HPE-100 system with coated capillaries (20 cm × 25 μm). Samples were dissolved in 0.1 M Na_2HPO_4 buffer, pH 2.5. The analysis was run at 12 kV and the effluent was monitored at 200 nm.

Size-exclusion chromatography

Size-exclusion chromatography was conducted at room temperature on a Superose-12 HR 10/30 (Pharmacia-LKB) column, eluted at a flow rate of 230 μL/min with 20 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl. Effluent from the column was monitored at 280 nm. Calibration of the Superose column was obtained with a mixture of bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anydrase (29.0 kDa), myoglobin (15.9 kDa), RNase (13.7 kDa), and aprotinin (6.5 kDa).

Amino acid analysis and sequencing

For amino acid determination, the protein/fragment samples were hydrolyzed in gas phase with 6 N HCl containing 0.1% phenol for 1 h at 150 °C utilizing the Pico-Tag workstation (Waters, Milford, California). Free amino acids were reacted with phenylisothiocyanate and the resulting PTC-derivatives analyzed by RP-HPLC (Heinrikson & Meredith, 1984) on a Pico-Tag C18 column (3.9 × 150 mm; Waters) utilizing a Perkin-Elmer liquid chromatograph (model 410-Bio) connected to an automatic injection system (Perkin-Elmer, model ISS-101). Automatic Edman degradation was performed with an Applied Biosystems protein sequencer (model 477A) equipped with an on-line analyzer (model 120A) of PTH-derivatives of amino acids.

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Mass determination

Mass spectra were recorded using a time-of-flight matrix-assisted laser desorption mass spectrometer (Reflex, Bruker). A mixture of 5 μL of a 10 μM analyte solution and 5 μL of 0.01 mM sinapinic acid in 20% acetonitrile was applied to the metallic probe tip and dried in vacuo. A pulsed laser beam (337 nm, 3 ns) was focused on the sample and the ions were accelerated with a linear potential of 30 kV. The mass spectrometer was calibrated with horse heart myoglobin utilized as an internal standard. Raw data were analyzed and stored using X-Mass software provided by Bruker.

CD

CD measurements were conducted on a Jasco (Tokyo, Japan) J-710 spectropolarimeter utilizing 1-mm or 5-mm pathlength quartz cell in the far-UV and near-UV region, respectively. The instrument was calibrated with ammonium d-10–camphorsulfonic acid. The mean residue ellipticity $[\theta]$ (deg $\times$ cm^2 $\times$ dmol^{-1}) was calculated from the formula $[\theta] = (\theta_{obs}/10) \times (\mathrm{MRW}/lc)$, where θ_{obs} is the ellipticity in deg, MRW is the mean residue molecular weight, c is the protein concentration in g/mL, and l is the pathlength of the cuvette in cm. The concentration of RNase and its nicked forms was determined spectrophotometrically by using a molar absorbance of 9.8×10^{-3} cm^{-1} M^{-1} at 278 nm (Lang & Schmid, 1986) and MRW was taken as 110. Thermal unfolding was monitored by recording the decrease of the CD signal at 220 nm as a function of sample temperature. Denaturation experiments for native and nicked RNase were conducted at a protein concentration of 0.05 mg/mL in 50 mM Na_2HPO_4 buffer, pH 7.0, containing 0.1 M NaCl, using a rectangular 1-cm pathlength quartz cuvette heated by a water circulating bath (Neslab, Newington, New Hampshire) at a heating rate of 50 °C/h. CD and temperature data were recorded and elaborated by a computer program provided by Jasco. Analysis of far-UV CD spectra to estimate the percent of protein secondary structure was performed with a computer program provided by Jasco and based on the method of Chen et al. (1972).

NMR spectroscopy

NMR samples in aqueous solution were prepared by dissolving the protein at 2 mM concentration in H_2O/D_2O 9:1. For samples in TFE, the appropriate volume of deuterated TFE (Cambridge Isotope Chem.) was added to the aqueous NMR sample. TSP was used as an internal reference. Two-dimensional ^{1}H-NMR experiments were performed on a Bruker AMX-600 spectrometer. The two-dimensional spectra were acquired in the phase-sensitive mode using the time-proportional phase incrementation technique (Marion & Wüthrich, 1983) with presaturation of the water signal. TOCSY (Bax & Davis, 1985) spectra were performed using the standard MLEV17 spinlock sequence and 75-ms mixing time. NOESY (Kumar et al., 1980; Macura & Ernst, 1980) spectra were recorded using standard phase-cycling sequences with a mixing time of 100 ms. The size of the data matrix was 2,048 $\times$ 512 words in f_2 and f_1, respectively, and was zero-filled up to 4,096 $\times$ 1,024 prior to Fourier transform.

Enzymatic assays

Assays of the enzymatic activity of RNase and its nicked forms Th1 and Th2 were determined at 25 °C following the increase in absorbance at 287 nm of a 3-mL solution of 0.15 M cytidine 2′,3′-phosphate in 0.1 M Tris acetate buffer, pH 7.0, after addition of 20 μg of enzyme (Crook et al., 1960).

Supplementary material in Electronic Appendix

Figures showing the amino acid sequence of RNase and the RP-HPLC chromatograms of reduced and S-carboxamidomethylated samples of RNase Th1 and Th2, as well as a table with the amino acid composition, N-terminal sequence, and molecular mass of the polypeptide chains constituting RNase Th1 and Th2, can be found in the Electronic Appendix.

Acknowledgments

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