Transfer RNA Cleavages by Onconase Reveal Unusual Cleavage Sites*

Received for publication, April 25, 2005, and in revised form, February 15, 2006. Published, JBC Papers in Press, February 23, 2006, DOI 10.1074/jbc.M504488200

Avvaru N. Suhasini1 and Ravi Sirdeshmukh2
From the Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Onconase, a protein from amphibian eggs and a homologue of pancreatic ribonuclease (RNase) superfamily, is cytotoxic, exhibits antitumor and antiviral activity, and is in phase III clinical trials. It has been shown to predominantly target cellular tRNA on its entry into mammalian cells (Saxena, S. K., Sirdeshmukh, R., Ardelt, W., Mikulski, S. M., Shogen, K., and Youle, R. J. (2002) J. Biol. Chem. 277, 15142–15146). Cleavage site mapping using natural tRNA substrates, in vitro, revealed predominant cleavage sites at UG and GG residues. Cleavages at UG or the D-arm of the tRNA are consistent with the known base specificity of onconase. However, predominance of cleavages at selected G–G bonds is unusual for a homologue of pancreatic RNases. Interestingly, in at least three of the four tRNA substrates studied, the predominant cleavages mapped in the triplet UGG located in the context of the variable loop or the D-arm of the tRNA. The cleavage specificity of onconase observed by us thus indicates another special feature of this enzyme, which may be relevant to its cellular actions.

The classical function of pancreatic RNases, a typical member of the group being bovine pancreatic RNase (RNase A),3 is to process and dispose of RNAs in the digestive tissues. However, it is now well accepted that this group of RNases serves more than mere digestive function, with several members shown to possess special biological properties. They include the mammalian members bovine seminal RNase, angiogenin, esophagin-derived neurotoxin, esophagin-derived cationic protein, and onconase, an amphibian homologue (2–5). Onconase, a protein from the eggs of Rana pipiens, originally discovered for its antitumor activity and later found to be homologous to pancreatic RNases (6), has been extensively studied for its cytotoxic property and consequent clinical applications. When incubated with mamalian cells in culture, the protein exerts cytotoxic and cytostatic effects (7), exhibits antitumor activity in vivo, and is presently in phase III clinical trials (8–10). At concentrations non-toxic to cells, it was found to inhibit HIV-1 replication in HIV-infected human H9 leukemia cells (11, 12) and may thus exhibit antiviral action in general. The mechanisms of cytotoxicity or the antiviral action as well as the specific molecular targets of the enzyme have been the subject of investigations by several groups (1, 13–16). Cytotoxicity seems to result from targeting the protein synthesis machinery after the enzyme enters the cells. Several known proteinaceous toxins of plant or bacterial origin inhibit protein synthesis by targeting tRNA of ribosomes (17–19). On the other hand, bacterial toxin colicin E5 specifically targets tRNAs (tRNAHis, tRNAAsp, tRNAArg, and tRNAThr; see Ref. 20) carrying modified guanosine, queuosine, at the wobble position followed by U. The antitumor RNase from Escherichia coli is another nuclease that specifically cleaves tRNA19–23 in T4-infected cells (21). Angiogenin, a pancreatic RNase homologue, was found to degrade tRNA when injected into frog oocytes (22), and we have earlier reported that the molecular target of onconase is also tRNA (1). However, the basis of this specificity toward tRNA has not been understood. When tried with synthetic substrates, onconase predominantly cleaved dinucleotides UpG and CpG (22–26). Our present studies indicate that the cleavages by onconase in several tRNAs involve selective G–G sites in addition to U–G and C–G dinucleotide bonds. Interestingly, the cleavage sites involving G–G locate in the variable or the D-arm region of the tRNA. As a member of the RNase A superfamily of proteins, onconase thus exhibits cleavage specificity not known for this group of enzymes. The specific cleavages by onconase in tRNA may give further insights into its biological effects.

EXPERIMENTAL PROCEDURES

Materials—Onconase was a gift from Richard Youle’s laboratory at the NINDS, National Institutes of Health, Bethesda, MD and Alfa Cell Corp., Bloomfield, NJ. RNase A, E. coli tRNA19–23, tRNAVal, yeast tRNAAsp, and E. coli tRNAThr were obtained from Sigma; T4 RNA ligase was from New England Biolabs, Beverly, MA. The RibomaxTM large scale RNA production system SP6 was from Promega, Madison, WI, and the RNA decamer marker was from Ambion, Austin, TX. The nucleotide removal kit and gel extraction kit were from Qiagen, Valencia, CA.32P-labeled cytidine 3′,5′-bisphosphate was prepared by Jonaki, Hyderabad, India.

Preparation of Rabbit Reticulocyte Lysates—Reticulocyte lysates were prepared as described in Ref. 27. New Zealand White male rabbits (2–3-months-old) were made anemic by subcutaneous injection of 1% acetyl phenyl hydrazine in water for four consecutive days. Five days after the last injection, blood was collected in precooled heparinized tubes. The blood was centrifuged at 3000 X g for 5 min at 4 °C, and supernatant was discarded. The cell pellet was washed three times (at 500 X g for 5 min each time) with buffered saline (130 mM NaCl, 10 mM HEPES, pH 7.2, containing 5 mM glucose, 7.5 mM MgCl2, and 5 mM KCl). The supernatant and white buffy coat present over the cell pellet were carefully removed. The packed volume of the cell pellet (red blood cells) was noted, and an equal volume of ice-cold deionized water was added to lyse the red blood cells. The lysed red blood cells suspension was centrifuged at 10,000 X g for 20 min at 4 °C. The supernatant was collected, and aliquots were stored at −80 °C until further use.

Preparation of RNA—RNA was isolated from the rabbit reticulocyte lysates as follows. A known volume of the lysates was diluted 100 times in buffer A (20 mM Tris-HCl, pH 7.4, 4 mM MgOAc, 100 mM KCl, 5 mM dithiothreitol), solubilized with 1% sarkosyl, and extracted with water-saturated phenol and chloroform. RNA from the aqueous phase was precipitated with an equal volume of isopropl alcohol. The RNA pellet

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 A Senior Research Fellow of the Council of Scientific and Industrial Research, Government of India.
2 To whom correspondence should be addressed. Tel.: 91-40-27192561; Fax: 91-40-27160311; E-mail: ravi@ccmb.res.in.
3 The abbreviations used are: RNase A, bovine pancreatic RNase; HIV, human immunodeficiency virus; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.
was washed with 70% ethyl alcohol, dried, and dissolved in RNase free water.

For the preparation of 28 S or 18 S rRNA, total RNA isolated from 100 μl of reticulocyte lysates as described above was centrifuged in the cold at 100,000 × g for 4.5 h through a 4.4-ml 5–20% discontinuous sucrose gradient in 0.1 M NaOAc, pH 5.2, containing 0.1 M NaCl and 0.001 M EDTA. 200-μl fractions were collected from the top of the gradient and checked for the respective RNA species by agarose gel electrophoresis. Fractions containing specific RNA species were pooled, and RNA was precipitated from the pooled fractions with isopropyl alcohol as above.

Cloning of tRNAPhe.m and Transcription in Vitro—tRNAPhe.m corresponded to the sequence of yeast tRNA^Phe^ in which residues U41 and A29 were replaced by A and U, respectively. This represents the compensatory mutation U41A,A29U, as shown in Fig. 7a. Both strands of DNA corresponding to the sequence of tRNA^Phe^ (along with NcoI or SacI restriction enzyme sites at the ends), were synthesized by DNA/RNA synthesizer 394 (Applied Biosystems). The synthesized oligonucleotides were purified using OPC cartridges (Applied Biosystems). The complementary oligonucleotide pairs (10 μg of each) were first denatured at 90 °C for 5 min and allowed to anneal at room temperature for 1 h. The annealed product was cloned into pGEM T Easy vector (Promega) under Sp6 promoter using SacI and NcoI sites. The DNA sequence of the clone was verified using a 3100 DNA sequencer (Applied Biosystems). The DNA corresponding to the sequence of tRNAPhe.m, along with NcoI or SacI restriction enzyme sites, was synthesized by DNA/RNA synthesizer 394 (Applied Biosystems). The DNA cloned into pGEM T Easy vector was used to transcribe tRNAPhe.m in vitro using the Ribomax™ large scale RNA production system SP6, as per the manufacturer’s protocol (Promega). The in vitro synthesized transcript was purified by phenol extraction and precipitated with isopropyl alcohol.

All natural and in vitro transcribed tRNAs were 3′ end-labeled with 32P-labeled cytidine 3′,5′-bisphosphate, using T4 RNA ligase as described in Ref. 28. Labeled RNA was purified using nucleotide removal kit according to the manufacturer’s protocol. For the purpose of RNase reactions, the specific activity of the labeled RNA was adjusted to 10^6 cpm/μg by adding the appropriate amount of unlabeled tRNA.

RNase Reactions and Analysis—For studying the activity of RNase A and onconase, ~130 ng/μl RNA was incubated with specified enzyme concentrations in 15 μl of assay buffer (10 mM Tris-HCl, pH 7.4, containing 5 mM dithiothreitol and 0.1 mM NaCl) at 30 °C for 15 min. In the experiments with 3′ end-labeled tRNA, 160 ng/μl tRNA containing 10^5 cpm radioactivity was incubated with specified enzyme concentrations, and the reactions were carried out in 6 μl of assay buffer as above unless described otherwise (see Fig. 4b). The molar ratio of tRNA substrate (the substrate in most of the reactions) to onconase, ranging between 2 and 10, depends upon the concentration of the enzyme used. In the experiment with rRNA substrates, this ratio was more than 100. However, it should be noted that with macromolecular, long chain RNA substrates (including tRNAs), the number of cleavable phosphodiester bonds is much higher than the number of molecules per se, and this should be taken in account when considering enzyme substrate ratios.

In experiments with purified RNA or reticulocyte lysates, total RNA was phenol-extracted at the end of the reactions and analyzed in 1.5% agarose gels. In other reactions, RNA was analyzed by electrophoresis as described under “Experimental Procedures.” The arrow indicates the RNA band representing major cleavage products(s) of onconase.
device. RNase T1-reacted tRNA and a ladder generated by alkaline hydrolysis of the same tRNA were used for assignment of sequence. RNase T1 cleaves 3' to G. Therefore every labeled fragment generated in the reaction with T1 would extend from the labeled 3' end to the nucleotide 3' to every G in the sequence. Partial hydrolysis of the labeled RNA was carried out with 0.1 M NaOH containing 10 mM EDTA at 37 °C for 5 min. This RNA was used in the sequencing gels to get the size ladder. Alternatively, in reaction with in vitro transcribed tRNAphe.mRNA, RNA decamer markers (Ambion) labeled at their 5' end with [γ-32P]ATP using polynucleotide kinase were used to define molecular sizes.

Mass Spectrometry of Onconase—The homogeneity of onconase preparation used in our study and its molecular mass was confirmed by MALDI-TOF spectrometry (Voyager DE STR, Applied Biosystems) and is shown in Fig. 1.

RESULTS

When incubated with mammalian cells in culture, onconase was found to enter cells (13, 15) and to selectively cleave tRNA with no detectable degradation of the rRNA (1). The simplest explanation offered for this specificity of onconase toward its substrate was based on the possible protection of rRNA by bound proteins. This was also supported with the reaction of onconase on rabbit reticulocyte lysates spiked with free E. coli RNA. In several experiments, we observed that the free RNA was degraded at much lower enzyme concentration than the concentration required for degrading rRNA of the ribosomes, the difference between the two concentrations being as much as 100-fold (not shown). Even RNase A would show some difference toward such substrate forms, but the behavior of onconase was very striking. Intrigued with this, we were interested to study whether the specificity was only due to apparent protection of the RNA by bound proteins or was driven by any sequence or structural feature of the substrate. We have tried to answer this question by comparing the action of RNase A and onconase on purified rRNAs and purified tRNAs with that on the RNA in reticulocyte lysates. We have also mapped the cleavage sites of onconase in different tRNAs (model substrates) labeled at their 3' ends, using RNA sequencing approach.
**Onconase Specificity**

action of RNase A and onconase on 28 S or 18 S rRNA purified from reticulocyte lysates and on reticulocyte lysate directly. In the assays with purified 28 S and 18 S rRNA (Fig. 2, a and b, respectively), 10–20 pM RNase A or 25 nM onconase was required for detectable degradation of the substrates, and both the enzymes degraded rRNA. The difference in the concentration of the two enzymes observed for minimum detectable degradation was consistent with the relative differences in their catalytic activity described earlier (about 1000-fold difference, see Ref. 23). We then studied the action of onconase and RNase A, as a function of enzyme concentration, on the RNA in rabbit reticulocyte lysates (Fig. 2c). With RNase A, about 40 pM enzyme was adequate to cause detectable degradation, and rRNA was clearly the first target. Since the degradation products of RNAs would overlap with tRNA, it was difficult to precisely state at what concentration the degradation of tRNA occurred. In the case of onconase, tRNA was found to be the preferred substrate and not rRNA; degradation of tRNA could be detected with 50 nM enzyme, and degradation of rRNA, if any at all, could be detected only at 4–8 μM onconase. Thus the difference in the enzyme concentration required for detectable degradation of each of the two substrates, tRNA and rRNA of the ribosomes, was about 100-fold. Together, these results support the earlier observation (see Ref. 1) that the difference in the action of two enzymes was seen only when the reaction occurred in cells or in reticulocyte lysates; onconase differed from RNase A in that it preferentially degraded tRNA. To understand the basis of this specificity of onconase further, we compared the action of onconase and RNase A on purified tRNA^Phe_. As seen in Fig. 3, 200–500 pM RNase A cleaved tRNA^Phe_, yielding a number of products of different sizes, whereas with increasing concentrations of onconase (0.25–6 μM), the reaction progressively resulted in degradation product(s), moving as one major band on electrophoresis (shown by the arrow in the figure). The major band corresponded to the size of about 35–40 nucleotides, suggesting that the major site(s) of cleavage were in the middle part of the molecule. Other cleavage products were relatively low in intensity. It may be observed that the lowest concentration of onconase required for detectable degradation of the free tRNA in this experiment was higher than that required for degrading tRNA in the reticulocyte lysate (compare Fig. 2c, 50 nM with Fig. 3, 250–500 nM). This difference in the activity may be due to its higher stability in the presence of the proteins of the reticulocyte lysates or the higher activity of the enzyme toward tRNA complexed in the translational machinery in the lysates. Virtual resistance of protein bound ribosomal or polysomal RNA to onconase over a wide range of enzyme concentrations enough to degrade tRNA or a limited number of cleavages seen with the enzyme in tRNA substrate is interesting. Together, these observations suggest specific target sites for onconase and selectivity of cleavages by the enzyme in this substrate. To identify exact cleavage sites, we labeled tRNAs at their 3′ end with 32P-labeled cytidine 3′,5′-bisphosphate and determined the termini of the cleavage products by RNA sequencing approach. Cleavage site mapping in tRNA^Phe_ is shown in Fig. 4. In the experiment shown in Fig. 3, degradation was detectable at 0.5 μM concentration of the enzyme. Therefore even in the experiment shown in Fig. 4, we used enzyme concentrations ranging from 0.5 to 6.0 μM. Species B in Fig. 4a was a predominant cleavage product observed at this lowest onconase concentration, which remained so at higher enzyme concentrations. At increasing enzyme concentrations, additional cleavage products, species A, E, and I, were also seen. Species A, I, and E resulted from cleavages 3′ to pyrimidine as observed for onconase with synthetic substrates (23–26). Other cleavages seen were less intense. Interestingly, species B corresponded to a cleavage between two purines G42-G43. Fig. 4b, represents reactions with tRNA^Phe_ incubated with 0.5 μM onconase for specified periods up to 30 min. Virtually no change in the cleavage pattern was observed over increasing time periods. The relative intensities of the degradation products observed at the shortest incubation time (5 min) are shown in Fig. 4c. Species B accounted for more than 50% of all the degradation products. The intensity of any other single cleavage product was found to be ~10% of the total or less. In the control reaction with RNase A (Fig. 5a) and tRNA^Phe_, the cleavages detected did not correspond to any of the major cleavages observed with onconase. Similarly, in another control reaction, denatured tRNA^Phe_ was reacted with onconase, and the products were studied (Fig. 5b). Cleavages observed with undenatured tRNA^Phe_ (Fig. 4a) were detected even with the denatured substrate. However, no significant preference for the cleavage sites was exhibited by the enzyme.

Fig. 6 represents an experiment with onconase and additional tRNAs, namely tRNA^Lys_, tRNA^fMet_, and tRNA^Val_. Fig. 6, a and b, show the reac-
Onconase specificity

FIGURE 6. Onconase cleavage site mapping in tRNA\textsubscript{Lys}, tRNA\textsubscript{fMet}, and tRNA\textsubscript{Val}. In a, 0.16 \mu{g}/\mu{l} 3’ end-labeled tRNA\textsubscript{Lys} was incubated with specified concentrations of onconase (Onc) or RNase T1, in a 6-\mu{l} reaction volume, for 15 min at 30°C. Identical aliquots of the reaction mixture were directly loaded on a 12% polyacrylamide sequencing gel, and electrophoresis was carried out under denaturing conditions. Other details are as in Fig. 4, b, an experiment done as in panel a except that the sequencing gel run was longer to resolve species A and B. In c and d, 3’ end-labeled tRNA\textsubscript{fMet} or tRNA\textsubscript{Val}, respectively, was reacted with onconase or RNase T1 using the same conditions as described in panel a, and the cleavage products were analyzed on a 12% polyacrylamide sequencing gel. The box in panel d shows a section of the gel from an independent reaction and the sequencing gel run carried out for better resolution of the bands in the region of species B.

Thus it was observed that with each of the tRNAs examined for onconase action, cleavage at G–G bonds was a major cleavage made by the enzyme. Further, in three of these tRNAs (tRNA\textsubscript{Phe}, tRNA\textsubscript{Lys}, and tRNA\textsubscript{fMet}), the G–G cleavages are included in the triplet UGG, suggesting the importance of the UGG context for cleavage specificity. To examine the role of UGG in cleavage site susceptibility, we cloned a mutant tRNA\textsubscript{Phe} gene sequence under SP6 promoter by replacing U41 with A (in the cleavage site U41-G42-G43) along with a compensatory change A29 to U to retain base pairing and transcribed it \textit{in vitro}. The anticipated structure of the mutated transcription product, tRNA\textsubscript{Phe}\textsubscript{m}, and the corresponding nucleotide positions are shown in Fig. 7a. The reaction of this tRNA\textsubscript{Phe}\textsubscript{m} with onconase is shown in Fig. 7b. In this reaction, we looked for the cleavages at the mutated site. The construct carries additional nucleotides from the vector region flanking the actual tRNA sequence (37 residues on the 5’ end and 5 residues on the 3’ end). We therefore used labeled RNA size markers in the electrophoresis run to identify cleavage positions corresponding to species A and B that were observed with the natural tRNA\textsubscript{Phe} as shown in Fig. 4a. No detectable cleavage was observed at the mutated cleavage site positions, presumably due to the change of U41-G42 to A41-G42, thus supporting the role of UGG in cleavage site specificity. There was, however, a cleavage detected at an adjacent G–G site not seen strongly in the natural tRNA\textsubscript{Phe}. In the absence of the original preferred cleavage site, it is likely...
Onconase Specificity

a. tRNA\textsuperscript{Phe.m} transcript with mutations U41A and A29U in the yeast tRNA\textsuperscript{Phe} sequence.

b. PAGE gel showing cleavage products of tRNA\textsuperscript{Phe.m} transcript with onconase. The reaction details are as in Fig. 4.
that this adjacent site is accessible in the mutant. There were also additional cleavages in the 5' end region of the tRNA, although not very strong (see "Discussion" below).

**DISCUSSION**

Pancreatic RNase superfamily is an interesting family of proteins. RNase A, the representative member of the family, is extensively studied both for understanding protein structure as well as for understanding enzyme action (29). The superfamily also includes members that exhibit unusual but interesting biological properties. Bovine seminal RNase, eosinophil-derived neurotoxin, eosinophil-derived cationic protein, and onconase, the amphibian homologue, are cytotoxic (2–5), whereas another homologue angiogenin from mammalian solid tumors has been shown to aid blood vessel formation and thus exhibits organogenesis activity (30). Onconase is also reported to exhibit anti-HIV activity and thus carries special significance as an antiviral RNase (11, 12). Inside mammalian cells, onconase shows preference for cellular tRNA (1), and this may be important for its antiviral action. However, tRNAs may not be the only cellular target for onconase, and rRNA or mRNA may also be cleaved under some conditions or with different enzyme concentrations. Preliminary experiments with *in vitro* transcribed mRNAs indicate selective cleavages even in these substrates. However, we do not know whether the same nucleobase specificity is exhibited when the enzyme cleaves rRNA or mRNA.

Virtual resistance of protein bound ribosomal or polysomal RNA to onconase over a wide range of enzyme concentrations used or restricted cleavages exhibited by the enzyme in tRNA substrates is quite striking. Together, these observations suggest selectivity of cleavage sites for the enzyme. Out of all major and minor cleavages of onconase identified, nine in tRNA^Phe^, six in tRNA^Lys^, four in tRNA^fMet^, and five in tRNA^Val^, some seemed to be major. Species B corresponded to such major cleavage. Other cleavages were apparent as the enzyme concentration increased. Earlier studies on onconase action on synthetic substrates (23–26) have suggested a preference for UpG (U binding in the B1 sub-site and G in the B2 sub-site of the substrate-binding site of enzyme). Out of the intense cleavages observed, species A resulted from

---

**Figure 8. The cleavage sites of onconase in tRNAs.** a, cleavage site positions are shown in two-dimensional cloverleaf structures (32) of tRNA^Phe^, tRNA^Lys^, tRNA^Val^, and tRNA^fMet^ as observed in Figs. 4 and 6. Cleavage at G–G sites generating species B in respective tRNAs is shown with solid arrows, and other cleavages are shown with arrowheads. Residues involved in tertiary interactions are also shown. b, the proximity of the variable loop and the D-arm, the sites of the major cleavages, is illustrated in the diagrammatic representation of the tRNA three-dimensional (3-D) structure.

---

The reaction mixture was loaded on 12% polyacrylamide sequencing gel run and processed as in Fig. 4. The observed or expected positions of the cleavage products (as in Fig. 4) were identified with reference to OH^- and G ladder, and in addition, to radioactively labeled RNA decamer size markers run in the same gel. The dotted lines for species A and B represent their expected positions. The arrow indicates the plausible position of onconase cleavage site. The tRNA sequence is numbered from the residue corresponding to the first 5' nucleotide of the natural tRNA. The RNase T1 reaction or alkaline hydrolysis of tRNA proceeded to a greater extent in this experiment.
Onconase Specificity

the cleavage 3′ to pyrimidine within the dinucleotide U–G. Cleavage at U–G, generating species A, and other cleavages such as those involving residues C–G, are consistent with the base specificity already reported for onconase. Surprisingly, a major cleavage in each of the tRNA substrates (species B) involved G–G dinucleotides, G42-G43 in tRNA^{Phe}, G45-G46 in tRNA^{Lys}, and G19-G20 in tRNA^{Met} and in tRNA^{Val}. How these bases interact preferentially with the substrate-binding sites of the enzyme is not clear at this point.

Onconase shares 30% sequence homology with RNase A as well as the active site residues. Three of the four disulfide bonds present in RNase A are also present in onconase. Onconase, however, differs from RNase A in some of the sequence and structural features, which make it different from the latter in its enzymatic and biological behavior (see Ref. 23 and references therein). For example, Gly-88 in RNase A responsible for contact with RNase inhibitor is absent in onconase, and this presumably contributes to its inhibitor resistance and cytotoxicity (31). Its N terminus is blocked by pyrogallinic acid and may have implications on its variant catalytic properties as well as cytotoxicity (23, 25). Here we show that onconase cleaves predominantly at G–G sites not known for the pancreatic RNase homologues.

Bacterial toxin Colicin E3 inhibits protein synthesis by a specific cleavage in the Shine Dalgarno sequence of the 16 S rRNA (17). Colicin E5, with no sequence homology with E3 but having ribonuclease activity, specifically targets a modified base at the Wobble position in a select group of tRNAs (20). Another ribonuclease, present in latent form in E. coli and activated on T4 infection, specifically cleaves tRNA^{UAS} 5′ to the Wobble base (the anticodon RNase, see Ref. 21). Thus some RNases are known to cleave tRNAs in functionally important regions. Interestingly, in tRNA^{Phe}, tRNA^{Lys}, and tRNA^{Met}, the major cleavages of onconase occur within the triplet UGG, as seen in the respective two-dimensional cloverleaf structures of these tRNAs (Fig. 8); these sites are located in the context of the variable loop (tRNA^{Phe} and tRNA^{Lys}) or the D-arm (tRNA^{Met}) involved in the tertiary interactions of the tRNAs. UGG triplets are not present elsewhere in the sequence of these tRNAs. In tRNA^{Phe} and tRNA^{Lys}, the D-arm U_{17}GG is modified and present as D_{17}GG. Cleavages were detectable in the D-arm in these substrates (not mapped precisely) but apparently not predominant as revealed by the intensities of the cleavage products. Thus these sites in tRNA^{Phe} and tRNA^{Lys} seem to be weak targets, if at all, for onconase action, suggesting their relative resistance due to U17 to D17 modification. In tRNA^{Val}, the corresponding site present as D_{17}GG is still a good target for onconase as evident from a strong cleavage. However, the cleavage occurred not within but 3′ to D_{17}GG (i.e. at G19-G20). The present results do not permit a clear explanation for this observation.

Pancreatic RNase homologues usually cleave single-stranded RNA. Therefore it may be argued that the susceptibility of the major cleavage sites located in the variable loop or the D-arm regions in the respective tRNAs may be due to the absence of base-paired structures. However, this was not apparent in the reaction with RNase A. Thus single strandedness per se may not be the factor responsible for the accessibility of the cleavage sites, and the triplet UGG may have a certain role in the cleavage site specificity shown by onconase. An experiment with the mutant tRNA^{Phe} in which the cleavage site UGG was changed to AGG, further supports the role of UGG as the mutation resulted in virtual absence of the cleavage at this site. Further, with denatured tRNA^{Phe} the same cleavages as in native tRNA^{Phe} were made by onconase but with no apparent predominance of one over the other. Together, this suggests that UGG triplet, although important, may not be the only determinant of specificity of the enzyme action, and additional structural elements present in the native structures of these RNAs may be important for preferential accessibility of the specific cleavage sites of onconase. The mutant tRNA^{Phe} also has U_{17}GG in the D-arm region; however, the cleavage observed in this region (not mapped precisely) was found to be quite weak, if present at all. It is possible that the native structural elements recognized by the enzyme for its preferential cleavages are not present in the mutant. It would thus be interesting to further investigate and understand the nature of onconase cleavage sites.

Unique cleavage specificities of enzymes such as anticodon nuclease or colicin E5 from bacteria are reported in the literature (20, 21). Onconase thus appears to be a rare vertebrate RNase known to exhibit preferred cleavages in structurally distinct regions of the tRNA, the variable loop (in tRNA^{Phe} and tRNA^{Lys}) and the D-arm region (in tRNA^{Met} and tRNA^{Val}), apparently guided by sequence and structural determinants. These cleavages may interfere with the structural integrity of the tRNA. Once the structure is lost as a result of the major cleavage, other cleavages may occur in a base-specific manner as observed with higher concentrations of onconase. Onconase is known more for its cytotoxic and antiviral action in mammalian cells. If UGG triplets are accepted as cleavage determinants, tRNA^{Phe} with UGG in the anticodon loop may be considered a potential target of onconase action in cells. On the other hand, the in vivo function of this protein in egg cells remains unknown. Its tRNA specificity has been discussed in relation to RNA interference-mediated processes in cells (33). The cleavage specificity of onconase thus opens new questions related to its biological function as well as its biomedical applications.

Acknowledgments—We thank Y. Ramadasu, Dr. M. R. S. Srinivas, and S. Sunil Kumar for participation in some initial pilot experiments, Meher Sultana for synthesizing the DNA sequences for tRNA^{Phe} clone, and Dr. C. S. Sundaram for mass spectrometry. Onconase was a kind gift from Dr. Richard Youle’s laboratory at NINDS, National Institutes of Health, Bethesda, MD, and was prepared at Alfa Cell Corp., Bloomfield, NJ.

REFERENCES