Escherichia coli RNase D
CATALYTIC PROPERTIES AND SUBSTRATE SPECIFICITY

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The catalytic properties of purified RNase D were examined. The enzyme requires a divalent cation for activity, and this requirement can be satisfied by Mg\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\). RNase D is most active at pH 9.1–9.5, but this optimum may reflect an effect on the substrate as well as the enzyme. A variety of RNAs were tested as substrates for RNase D. Alteration of the 3′-terminal base has no effect on the rate of hydrolysis, whereas modification of the 3′-terminal sugar has a major effect. tRNA terminating with a 3′-phosphate is completely inactive as a substrate. A variety of RNAs were tested as substrates for RNase D. Addition of tRNA increase the activity of the substrate. The rate of hydrolysis of intact tRNA is very slow compared to tRNAs containing extra residues or compared to tRNAs from which part of the -C-C-A sequence has been removed. Oxidation of the terminal sugar, reduction of the deoxyribose with borohydride, or removal of the terminal AMP from intact tRNA increase the activity of the substrate. Addition of a second -C-C-A sequence gives an active substrate indicating that the relative resistance of intact tRNA to RNase D hydrolysis is not due to the sequence per se but to the structural environment of the 3′-terminus. Studies of the mode of action of RNase D indicate that it is an exonuclease which initiates hydrolysis at the 3′-terminus and removes 5′-mononucleotides in a random fashion. The requirements of RNase D for interaction with nucleic acids and for hydrolysis of various RNAs and the relation of these properties to its possible role as a processing nuclease are discussed.

Experimental Procedures

Materials—Phosphodiesterase-treated \(^{32}P\)tRNA was prepared as described previously (7). Periodate oxidation, NaBH\(_4\), reduction, and alkaline phosphatase treatment of \(^{32}P\)tRNA were reported earlier (8, 9). tRNA-C-C-[\(^{14}C\)]A, tRNA-C-[\(^{14}C\)]C, tRNA-C-[\(^{14}C\)]U, and tRNA-C-[\(^{14}C\)]JU were synthesized using liver tRNA nucleotidyltransferase as described (7, 10). tRNA-C-C-A-[\(^{14}C\)]Cn was prepared as reported earlier (10). The preparation used in these studies contained an average of 3 extra CMP residues. tRNA-C-C-A-[\(^{14}C\)]Cn-[\(^{3}H\)]A was synthesized by addition of a single residue of [\(^{3}H\)]AMP to the latter substrate (10). Poly(A) and poly(U) were purchased from Miles Laboratories, Inc. Salmon sperm DNA was obtained from Sigma Chemical Co. E. coli 5 S RNA was a gift from Dr. B. Dudock, Stony Brook. Rabbit liver tRNA nucleotidyltransferase was purified as reported (11). Purification of RNase D is described in the accompanying paper (1).

Enzyme Assays—RNase D activity was determined as described in the accompanying paper (1) except that KCl was generally omitted. Details of individual experiments are described in the legends.

Results

Requirements for Hydrolysis by RNase D—Examination of the requirements for RNase D action was carried out with both \(^{32}P\)dtRNA\(^{1}\) or tRNA-C-C-A-[\(^{14}C\)]Cn as substrates. With either substrate, the only requirement identified was for the presence of a divalent cation (Table I). In the case of dtRNA this requirement could be satisfied by either Mg\(^{2+}\), Mn\(^{2+}\), or Co\(^{2+}\), with the latter cation being most effective in the concentration range examined (1–5 mM). Other divalent cations, such as Zn\(^{2+}\), Ca\(^{2+}\), Cu\(^{2+}\), and Mg\(^{2+}\) were ineffective or inhibited. Optimum activity in the presence of Mg\(^{2+}\) was obtained at 5 mM cation, with no further change in the range of 5–20 mM (data not shown). Using tRNA-C-C-A-[\(^{14}C\)]Cn as substrate, Mg\(^{2+}\) was the most effective cation, leading to about twice the activity observed with either Mn\(^{2+}\) or Co\(^{2+}\), when present at 5 mM (Table I). We have previously reported (7) that RNase D was inactive against poly(A) in the presence of Mg\(^{2+}\). Substitution of Mn\(^{2+}\) or Co\(^{2+}\) for Mg\(^{2+}\) did not affect the inability of RNase D to act on this substrate. We also have not observed any effect of the polyamine, spermidine, on the activity of RNase D.

Effect of Monovalent Cations—In view of the requirement of RNase II for K\(^{+}\), this cation was included in the standard assay mix used for purification of RNase D (1). However, we have subsequently found that monovalent cations are unnecessary for RNase D activity against tRNA or tRNA precursors, and the increased ionic strength resulting from addition of KCl actually inhibits the reaction (Fig. 1). A similar decrease in activity is observed with NaCl or NH\(_4\)Cl, confirming

1 The abbreviation used is dtRNA, phosphodiesterase-treated tRNA.
2 H. Cudny, R. Zaniewski, and M. P. Deutscher, unpublished observations.
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TABLE I

Divalent cation requirements of RNase D

| Additions | Concentration (mM) | Activity | dtRNA | tRNA-C-C-A-Cn | m mol/5 min |
|-----------|--------------------|----------|-------|---------------|-------------|
| None      |                    |          |       |               | 0.16        |
| EDTA      | 1                  |          |       |               | <0.02       |
| Mg$^{2+}$ | 1                  |          |       |               | 0.71        |
| Mg$^{2+}$ | 5                  |          |       |               | 1.49        |
| Mn$^{2+}$ | 1                  |          |       |               | 0.93        |
| Mn$^{2+}$ | 5                  |          |       |               | 1.64        |
| Co$^{2+}$ | 1                  |          |       |               | 1.62        |
| Co$^{2+}$ | 5                  |          |       |               | 1.91        |
| Zn$^{2+}$ | 1                  |          |       |               | 0.24        |
| Zn$^{2+}$ | 5                  |          |       |               | 0.10        |
| Cu$^{2+}$ | 1                  |          |       |               | <0.02       |
| Cu$^{2+}$ | 5                  |          |       |               | 0.03        |
| Ca$^{2+}$ | 1                  |          |       |               | 0.11        |
| Ca$^{2+}$ | 5                  |          |       |               | 0.08        |
| Hg$^{2+}$ | 1                  |          |       |               | <0.02       |
| Hg$^{2+}$ | 5                  |          |       |               | 0.02        |

FIG. 1. Effect of KCl on RNase D activity. Assays were carried out as described under "Experimental Procedures" with 58 μg of [$^{32}$P]dtRNA, 9 milliunits of RNase D, and the indicated concentration of KCl. Incubations were for 5 min at 37 °C.

that the inhibition is most likely an ionic strength effect.

Effect of pH—Optimal activity of RNase D was obtained in the pH range of 9.1-9.5 with either dtRNA (Fig. 2) or tRNA-C-C-A-Cn (not shown). Since RNase D is extremely sensitive to the conformation of the tRNA substrates (7), we have avoided studies at the alkaline pH optimum and have continued to use pH 7.5 in the studies reported here. In fact, the apparent pH optimum shown in Fig. 2 probably reflects a partial denaturation of the tRNA substrates above pH 9, making them better substrates (7). Above pH 9.5, inactivation of the enzyme may begin to play a role.

Substrate Specificity of RNase D—Using relatively crude preparations we showed previously that RNase D was most effective against tRNA substrates and was completely inactive against the synthetic polynucleotides, poly(A) and poly(U) (7). Identical results were obtained with the purified RNase D described here. In order to probe the structural requirements for RNase D action in more detail, we have prepared a variety of tRNA substrates which differ in their 3'-terminal structure and have tested the ability of these molecules to act as substrates or inhibitors of RNase D.

FIG. 2. pH activity profile of RNase D. Assays were carried out as described under "Experimental Procedures" with 58 μg of [$^{32}$P]dtRNA and 9 milliunits of RNase D, except that 0.1 M KCl was present. Incubations were for 5 min at 37 °C. Sodium acetate buffer (H), Tris-C1 buffer (M), and glycine-NaOH (L) were used in the various pH ranges as indicated.

FIG. 3. Role of the 3'-terminal base on tRNA. Assays were carried out as described under "Experimental Procedures" with either 39 μg of tRNA-C-[^4C]A, 39 μg of tRNA-C-[^4C]C, or 40 μg of tRNA-C-[^4C]U. Incubations were for 5 min at 37 °C with the indicated amounts of RNase D.
identity, is the more important determinant for RNase D action. This idea will be explored in more detail below.

Effect of 3'-Terminal Sugar or Removal of 3'-Terminal Residues—Further details of the specificity of RNase D action were examined using a series of \[^{32}P\]tRNAs which had been altered in the 3'-terminal sugar by periodate oxidation, borohydride reduction, or removal of the terminal nucleoside to generate a 3'-phosphoryl-terminated tRNA. In addition, these substrates were compared to tRNA-C-C and dtRNA (Table II). As we have noted previously using less pure preparations of RNase D (7), treatment of tRNA with snake venom phosphodiesterase leads to a large increase in its ability to act as a substrate (Table II). In this experiment, in which initial rates are being measured, dtRNA (average of 2 residues removed) is degraded close to 10 times as rapidly as intact tRNA. Similarly, tRNA-C-C is a good substrate for RNase D, although it should be noted that the removal of the 5' phosphate during preparation of this substrate may affect the activity of RNase D (7).

Effect of Additional Residues at the 3'-Terminus—Addition of extra residues to the 3'-terminus of tRNA, such as in the case of tRNA-C-C-A-[\(^{14}C\)]A, leads to a large increase in the rate of nucleotide hydrolysis by RNase D (Fig. 4). Removal of the radioactive residues from tRNA-C-C-A-[\(^{14}C\)]A proceeds about 20-30 times faster than removal of the terminal AMP residue from tRNA-C-C-A-[\(^{14}C\)]A. This observation emphasizes the role of the 3'-terminal structure of tRNA at the sugar moiety or by removal of nucleotide residues can greatly affect how well a tRNA will be hydrolyzed by RNase D.

Role of the -C-C-A Sequence—The studies with modified tRNA substrates indicate that a tRNA with an intact -C-C-A sequence is relatively resistant to the action of RNase D, whereas shortening, elongating, or modifying this sequence leads to tRNAs that are hydrolyzed by RNase D at greatly increased rates. The question arises whether the relative resistance of intact tRNA-C-C-A is due to the presence of the -C-C-A sequence per se or to a resistant conformation found in intact tRNA molecules. To answer this question, a second -C-C-A sequence was added to tRNA, and the rate of removal of the terminal AMP from the extra -C-C-A in tRNA-C-C-A-[\(^{14}C\)]A was compared to removal of the terminal AMP from intact tRNA-C-C-A-[\(^{14}C\)]A. As shown in Fig. 4, removal of the terminal residue from the extra -C-C-A sequence is much more rapid (about 40-fold) than removal of AMP from intact tRNA. This experiment indicates that a -C-C-A sequence itself confers no special resistance to RNase D, but rather, that the structural environment of the 3'-terminus is the determining factor.

Interaction of Nucleic Acids with RNase D—Although RNase D is quite specific with respect to its hydrolysis of different RNAs, it appears to be relatively nonspecific in its ability to interact with various nucleic acids. In the accompanying paper (1) it was already shown that intact tRNA, tRNA, and poly(A) each could protect RNase D against thermal inactivation, implying that each of these RNAs can bind to the enzyme. Furthermore, a variety of nucleic acids inhibit RNase D hydrolysis of tRNA-C-C-A-[\(^{14}C\)]A (Fig. 5). These include intact and partially degraded tRNAs, 5 S RNA, poly(U), and salmon sperm DNA. Surprisingly, poly(A), which could stabilize RNase D against thermal inactivation, did not inhibit within the concentration range tested. On the other hand, poly(A) was an effective inhibitor when tRNA-C-C-A-[\(^{14}C\)]A was the substrate, as were the other RNAs tested (data not shown). At the present time the anomalous behavior of poly(A) is not understood. Nevertheless, it is clear that a variety of nucleic acids, including those which are not substrates, can interact with RNase D. The relative affinities of different RNAs, as well as the structural features which determine whether an RNA will be a substrate for RNase D, are under investigation.

Mode of Action—Evidence has previously been presented (7) that the acid-soluble products of RNase D action are 3'-mononucleotides. We have confirmed these results using \[^{32}P\]tRNA and the purified enzyme. Since these data are identical with those reported earlier, we will not repeat them here. Furthermore, the aminoacylation studies described earlier (6) and the lack of action on \[^{32}P\]tRNA-C-Cp described in this paper support the conclusion that RNase D hydrolysis is initiated at the 3'-end of RNA molecules.

### Table II

| Substrate | Activity | Reaction Time |
|-----------|----------|--------------|
| Intact tRNA | 0.08 | 10 min |
| tRNA-C-Cp | <0.02 | 10 min |
| tRNA-oxidized | 0.11 | 10 min |
| tRNA-reduced | 0.27 | 10 min |
| tRNA-C-C-A | 0.66 | 10 min |
| dtRNA | 0.74 | 10 min |

* tRNA-oxidized tRNA oxidized with Na periodate.
* tRNA-reduced tRNA reduced with NaBH₄.
We have also suggested earlier (6), using tRNA-C-C-A-Cn as substrate, that RNase D acts randomly, falling off the RNA substrate after every hydrolytic event. The data in Fig. 6 show that this mode of action also applies to RNase D hydrolysis of tRNA-C-C-A. In this experiment the incorporation of [\(^{32}\)P]AMP into tRNA-C-C-A in the presence of RNase D, tRNA nucleotidyltransferase, and [\(^{14}\)C]ATP is measured. No incorporation is found in the absence of RNase D, whereas continued incorporation over a period of 30 min is observed in its presence. The finding that AMP incorporation occurs under these conditions indicates that RNase D must leave the 3'-terminus after removal of 1 residue in order to allow tRNA nucleotidyltransferase to replace the missing AMP moiety. Since the presence of CTP does not increase [\(^{14}\)C]AMP incorporation (Fig. 6), tRNA nucleotidyltransferase must act to replace AMP before RNase D can remove a CMP residue. RNase II, which is known to act processively (12), does not lead to [\(^{14}\)C]AMP incorporation under these conditions (Fig. 6). These results support a random mode of hydrolysis for RNase D.

**DISCUSSION**

The data presented here and elsewhere (6, 7) support the conclusion that RNase D is an exonuclease which initiates attack at the 3'-terminus of an RNA molecule and releases 5'-mononucleotides in a random fashion. However, in some experiments utilizing natural tRNA precursors, evidence for endonucleolytic cleavages also was obtained. Since these latter experiments were carried out at high enzyme to RNA ratios, the nonspecific degradation may be due to a low level of contamination by an endonuclease which becomes apparent under the conditions of these studies. Although we have not obtained any evidence for endonucleolytic cleavages under the conditions described here (about half-saturating for dsRNA), we have not eliminated the possibility that they are an intrinsic low level activity of RNase D.

The substrate specificity studies provide strong support for the notion that RNase D is sensitive to the conformation of the 3'-terminus of the RNA substrate rather than to its actual base sequence. This was demonstrated by the absence of any effect on RNase D activity upon changing the 3'-terminal nucleotide, and more dramatically, by the greatly increased hydrolysis of a -C-C-A sequence beyond the normal 3'-terminus compared to the same sequence in intact tRNA. Since tRNA molecules devoid of the normal 3'-sequence and those with extra residues beyond this sequence are both hydrolyzed rapidly by RNase D, some structural feature of intact tRNA must render the -C-C-A sequence in this molecule inaccessible to the catalytic site of the nuclease. At the present time we do not know if this is simply a matter of length from the start of the double-stranded aminoacyl stem of tRNA or an interaction of the 3'-terminus of intact tRNA with another part of the molecule. Perhaps, studies with other model substrates will resolve this question.

From these studies it is also clear that a variety of nucleic acids, including those that are not substrates, can interact with RNase D. However, the details of some of these interactions are not yet understood. Nevertheless, the results imply that substrate binding and recognition for catalysis are separate events. Similar mechanisms have been suggested recently for RNase M5 (13), tRNA nucleotidyltransferase (14), and aminoacyl-tRNA synthetases (15) and are probably a general feature of enzyme-nucleic acid interactions.

The substrate specificity of RNase D makes it well suited to be a 3'-processing nuclease. Its action on extra residues following the -C-C-A sequence would allow rapid processing of extraneous sequences, and its relatively poor activity on intact tRNA coupled with a random mode of hydrolysis would permit aminoacylation of the mature tRNA prior to degradation. Studies with mutants are in progress to determine whether RNase D actually functions as a processing enzyme in vivo.

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