Determination of the Catalytic Parameters of the N-terminal Half of Escherichia coli Ribonuclease E and the Identification of Critical Functional Groups in RNA Substrates*

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Ribonuclease E is required for the rapid decay and correct processing of RNA in Escherichia coli. A detailed understanding of the hydrolysis of RNA by this and related enzymes will require the integration of structural and molecular data with quantitative measurements of RNA hydrolysis. Therefore, an assay for RNaseE that can be set up to have relatively high throughput while being sensitive and quantitative will be advantageous. Here we describe such an assay, which is based on the automated high pressure liquid chromatography analysis of fluorescently labeled RNA samples. We have used this assay to optimize reaction conditions, to determine for the first time the catalytic parameters for a polypeptide of RNaseE, and to investigate the RNaseE-catalyzed reaction through the modification of functional groups within an RNA substrate. We find that catalysis is dependent on both protonated and unprotonated functional groups and that the recognition of a guanosine sequence determinant that is upstream of the scissile bond appears to consist of interactions with the exocyclic 2-amino group, the 7N of the nucleobase and the imino proton or 6-keto group. Additionally, we find that a ribose-like sugar conformation is preferred in the 5'-nucleotide of the scissile phosphodiester bond and that a 2'-hydroxyl group proton is not essential. Steric bulk at the 2' position in the 5'-nucleotide appears to be inhibitory to the reaction. Combined, these observations establish a foundation for the functional interpretation of a three-dimensional structure of the catalytic domain of RNaseE when solved.

RNaseE is a single-stranded-specific endoribonuclease (1–3) that is required for the rapid decay of many if not most transcripts in Escherichia coli (for reviews, see Refs. 4–6) and the correct processing of precursors of, for example, ribosomal and transfer RNAs (7, 8). RNaseE generates products terminating in a 3'-hydroxy and a 5'-phosphate (9) indicating that the reaction involves the attack of water on the susceptible phosphodiester bond followed by scission of 3'-oxygen-phosphorus bond. The RNaseE polypeptide consists of 1061 residues (Fig. 1, panel A). The N-terminal half (NTH) of RNaseE is sufficient for basal catalytic activity (10) that is able to support cell viability (11). The RNaseE-catalyzed hydrolysis of RNA in vivo is influenced by the C-terminal half (12–14), which contains an arginine-rich RNA-binding domain (10, 14–17) and several sites (18, 19) that serve in the assembly of the E. coli RNA degradosome (for reviews, see Refs. 20 and 21). The 3'-exonuclease polynucleotide phosphorylase, the RhlB helicase, and the glycolytic enzyme enolase are the other major components of this macromolecular complex (22, 23).

In general, it appears that endonucleolytic hydrolysis by RNaseE initiates the decay of transcripts in E. coli (for reviews, see Refs. 4–6). Moreover, it has been shown in vitro that RNaseE preferentially cleaves RNAs that have a 5'-monophosphate group (24). As primary transcripts in E. coli have a triphosphate group at their 5’ end and the RNaseE-catalyzed hydrolysis generates downstream intermediates that have a 5'-monophosphate group (9), the ability of RNaseE to assess the phosphorylation status of 5’ ends may ensure that after this enzyme has made the first cut in a primary transcript, it will tend to cut any sites downstream before starting on another intact transcript. The N-terminal half of RNaseE is sufficient to discriminate substrates that have a monophosphate group at the 5’ end (25, 26). There is evidence for “5’ end dependence” in vivo and it has been suggested that this facet of RNaseE-catalyzed cleavage may contribute to the finding that RNA decay occurs in general without the production of significant levels of intermediates in E. coli (27).

Polypeptides that have sequences similar to E. coli RNaseE have been predicted from approximately half of the eu-bacterial genomes that have been sequenced and in the plastids of plants (for review, see Ref. 28). In all of the cases that we know of, the sequence similarity covers at least part of the N-terminal half of E. coli RNaseE. Only some members of the RNaseE family are elaborated with N- and C-terminal extensions, and these vary greatly in length and have little sequence conservation (28). In the case of E. coli, it is known that the C-terminal domain is required for the normal rapid decay of mRNA in vivo (12–14) and it has been suggested that extensions serve to adapt the activity of the catalytic domains of RNaseE-related enzymes to mediate patterns of RNA processing and decay that are appropriate for the corresponding bacteria or plastid (14).

Oligonucleotide substrates synthesized using phosphorami-
dite chemistry are being used increasingly to study hydrolysis of RNA by RNaseE in vitro. In the first study of this enzyme that used oligonucleotide substrates, it was found that a deacarboxy- nucleotide (ACAGA | AUUUG) corresponding in sequence to the single-stranded region at the 5′ end of RNAi, the antisense regulator of ColE1-type plasmid replication, is cut at least as efficiently as the corresponding full-length transcript by RNaseE(29). It was subsequently found for the 9 S precursor of 5 S RNA that the “a” site (ACAGA | AUUUG) but not the “b” site (AUCAA | AUAAA) is cut efficiently (17). The b site in contrast to the other two, lacks a G nucleotide upstream within 1–2 nucleotides of the phosphodiester bond, which within the context of 9 S RNA is hydrolyzed. Moreover, the swapping of a G for the A at position 4 in the b site oligonucleotide was sufficient to confer efficient hydrolysis, whereas the swapping of a U for the G at position 4 in the a site blocked hydrolysis of the oligonucleotide substrate (17). The alignment of known RNaseE sites (e.g. Ref. 30) has revealed that many have a G nucleotide that is 5′ and is in close proximity to the susceptible bond. In light of the swapping experiments described above, this suggests that such positioned G nucleotides are of general importance as determinants of RNA hydrolysis in E. coli. The mechanism by which the b site within the context of a 9 S transcript is cleaved by RNaseE despite the absence of an upstream G remains to be determined. It is possible that the recruitment of RNaseE is regulated by some secondary structural element(s) (17) as suggested relatively early in the study of RNaseE (1, 30) or the distance between susceptible sequences and a monophosphate at the 5′ end (25).

From the above, it can be seen that an understanding of the recognition and hydrolysis of RNA by RNaseE will require a systematic analysis of the contribution of RNA sequence and structure, including the phosphorylation status at the 5′ end, the multiple domains within the C-terminal half of RNaseE, and the effects of other degradative component. With this in mind, we have developed an assay system described here that can have relatively high throughput while being sensitive and quantitative. We also describe the optimization of reaction conditions for the N-terminal catalytic half of RNaseE and the determination of Michaelis-Menten parameters for this enzyme. In addition, we have identified functional groups in the G determinant that are required for efficient hydrolysis and the importance of substrates being ribose. This work establishes a foundation for investigating the recognition and hydrolysis of RNA by the RNaseE family of enzymes.

**EXPERIMENTAL PROCEDURES**

**Synthesis of Oligonucleotide Substrates**—The oligoribonucleotides used in this study are shown in Table I. They were synthesized using nucleotide (ACAGA | AUUUG) oligonucleotide substrates, it was found that a decarboxy-nucleotide chemistry are being used increasingly to study hydrolysis of RNA by RNaseE with a decarboxy-linked tag at the C terminus was generated using a pair of primers with the following sequences: 5′-CATGCGATGAGAAGAT- GGTTAACACCG-3′ (start codon is in italics, and Nco site is underlined) and 5′-CGGATCTTTAGTGATGTATAGTGATGAT- GATGATCAGGCGGGTTCGGC-3′ (‘anti-stop codon is in boldface, and BamHI site is underlined). The PCR product was cut with NcoI and BamHI and inserted between the corresponding sites in the expression vector pET16b (Novagen). The resulting construct, called phiE529c, was then sequenced from both strands.

**Production and Purification of the N-terminal Half of RNaseE**—This was done using the pET expression and purification system (31–33). A DNA fragment encoding the first 529 amino acid residues of RNaseE with a decarboxy-linked tag at the C terminus was used in this study. The expression vector pET16b (Novagen) was used for NTH-RNaseE tagged at the C terminus was 26,920M. The final protein concentration was determined by the 280 nm absorbance. The extinction coefficient used for NTH-RNaseE tagged at the C terminus was 26,920 M 1 cm−1 as calculated using the ExPaSy Molecular Biology Server tool (http://expasy.org). The purity of the preparation was greater than 95% as judged by SDS-PAGE analysis.

**The Dependence of the Hydrolysis of RNA by RNaseE on pH**—The effects of pH on RNA hydrolysis was studied using a substrate concentration of 6 μM and an enzyme concentration of 1 nM. Under these conditions, the initial reaction rate approached first order. The reactions contained 10 mM MgCl2, 100 mM NaCl, 0.1 % (v/v) Triton X-100, 1 mM dithiothreitol, and 25 mM of a buffering agent. The following pH values were analyzed: 5.5, 6.0, 6.5 (MES-HCl), 7.0, 7.3, 7.6, 8.0 (Tris-HCl), 8.5, 9.0, 9.5 (Bio-Tris-propane-HCl), 10.0, and 10.5 (CAPS-HCl). The reactions were initiated by addition of an enzyme that was in a 20-fold excess over the substrate concentration. Reaction mixtures were incubated at 37 °C, and aliquots were taken at appropriate time points. Samples were quenched by mixing aliquots with an equal volume of 20 mM EDTA and analyzed by ion-pair reverse phase chromatography using a Dionex high pressure liquid chromatography system with an in-line fluorescence detector (Model RF-2000) and automated sample injector (Model ASI-100). Chromatograms were analyzed with the use of

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2 Callaghan, A. J., Grossmann, J. G., Redko, Y., Ilag, L. L., Moncrieffe, M. C., Symmons, M. F., Robinson, C. V., McDowell, K. J., and Luisi, B. F. (2003) Biochemistry, in press.
of Chromleon SP1 software, version 6.4. A Transgenomic DNA Sep® cartridge was equilibrated with 70:30 mixture of 2.5 mM tetrabutyl ammonium bromide, 1 mM EDTA, and 0.1% (v/v) acetonitrile (buffer A) and 2.5 mM tetrabutyl ammonium bromide, 1 mM EDTA, and 70% (v/v) acetonitrile (buffer B). RNA oligonucleotides were eluted with a gradient of 30–60% buffer B over 10 min. The system was calibrated using a 5-mer, p-BR5-Fl, which corresponded to the 3’ product of RNaseE hydrolysis but was synthesized chemically. Initial rates of reaction at the various substrate concentrations were determined by plotting the amount of product against time. Data from triplicate experiments were averaged and fitted to a double ionization equation (35) using KaleidaGraph software (Synergy Software, Reading, PA) as shown in Equation 1, where $K_{eq}$ and $K_{d}$ are acid dissociation constants of the free enzyme, $k_{cat}$ is the rate constant at a given pH, and $k_{m}$ is the maximal rate constant of the catalytically competent form of the enzyme.

The Optimization of Magnesium and Salt Concentrations—To establish the optimum level of MgCl$_2$, the concentration was varied from 0 to 150 mM. The reaction buffer for this experiment also contained 25 mM Bis-Tris-propane-HCl, pH 8.3, and 100 mM NaCl. The level of salt was optimized next using concentrations from 0 to 500 mM. This was done using reaction buffer that contained 25 mM Bis-Tris-propane-HCl, pH 8.3, and 15 mM MgCl$_2$.

Determination of Michaelis-Menten Parameters—RNaseE substrate p-BR10-Fl was incubated with 1 nM of the N-terminal half of RNaseE in 25 mM Bis-Tris-propane-HCl, pH 8.3, and 100 mM NaCl, 15 mM MgCl$_2$, 0.1% (v/v) Triton X-100, and 1 mM dithiothreitol. The substrate concentrations varied from 100 nM to 6 μM. Kinetic parameters were calculated by non-linear regression fitting of the data to the Michaelis-Menten equation as shown in Equation 2,

$$v = \frac{k_{cat}[S]}{K_m + [S]}$$

(Eq. 2)

where $v$ is the initial rate, [S] is the substrate concentration, and [E] is the total enzyme concentration.

Assay of RNaseE under Pseudo-first Order Conditions—The reaction conditions and assay procedures were as described above. The final substrate and enzyme concentrations were 10 and 100 nM, respectively. The experiments were done at least three times, and average data were plotted and fitted to the pseudo-first-order equation as shown in Equation 3,

$$P = S_0 \left(1 - \exp \left(\frac{k_{cat}[E]}{K_m[S]} t\right)\right)$$

(Eq. 3)

where $P$ is percentage of the product, $S_0$ is the initial percentage of substrate (100%), and [E] is the total enzyme concentration.

RESULTS

The Assay of RNaseE by High Pressure Liquid Chromatography Analysis of Fluorescein-labeled RNA—As an alternative to incorporating $^{32}$P at the 5’ end of oligoribonucleotides post-synthesis, we added fluorescein (Fl) to their 3’ end during chemical synthesis (for details of the RNAs used in this study, see Fig. 1). When required, a monophosphate (p) was added chemically to the 5’ end during synthesis to avoid the significant variation in efficiency that can occur when T4 polynucleotide kinase is used. In contrast to $^{32}$P-labeled substrates, we find that those with fluorescein can be kept for many months without detectable loss of label. Using a fluorescing imaging system, we have been able to detect bands in polyacrylamide gels that correspond to <10 femtomoles of fluorescein-labeled RNA. This is comparable to the sensitivity of detecting $^{32}$P-labeled RNA by phosphorimaging (36). We have now automated the resolution and detection of RNA in reaction mixtures using ion-pair reverse phase chromatography coupled with an in-line fluorescence detector (for details, see “Experimental Procedures”). As shown in Fig. 2, panel A, a 5-nucleotide 3’- (Fl)-labeled fragment produced by hydrolysis of p-BR10-Fl, a 10-nucleotide substrate corresponding in sequence to the RNaseE site at the 5’ end of RNAI from pBR322 (29), can be separated readily. Moreover, this system can separate oligonucleotide substrates that differ only in the presence or absence of a monophosphate at the 5’ end (data not shown). Quantitation was calibrated using p-BR5-Fl, a synthetic mimic of the 5-nucleotide hydrolysis product. The fluorescence response was linear over our working range of 40 fmol–10 pmol (Fig. 2, panel B). Furthermore, the amount of fluorescence from p-BR5-Fl was the same as that from equal amounts of p-BR10-Fl or a derivative p-BR13-Fl, which has three extra Gs at the very 5’ end (data not shown).

Optimization of Conditions for RNaseE Hydrolysis of Oligonucleotide Substrates—The reaction conditions for the NTH of RNaseE were optimized using a stepwise approach. First, the pH of the buffer was optimized and then the magnesium concentration and finally the concentration of NaCl (Fig. 3). The 3’-fluorescein-labeled substrate for all of these experiments

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2 All of the curve fitting was carried out using KaleidaGraph software, version 3.52 (Synergy Software).

3 J. A. Stead and K. J. McDowall, unpublished data.
was p-BR13-Fl. Preliminary experiments that used published conditions (29) to compare the hydrolysis of BR13 (radiolabeled at the 5'-end) with and without fluorescein at the 3'-end found that the rates of the reactions were comparable, suggesting that this label at the 3'-end does not have a significant effect on hydrolysis by the NTH of RNaseE (data not shown). The plot of the reaction rate against pH was bell-shaped (panel A), indicating that catalysis requires both unprotonated and protonated functional groups. By fitting the pH rate profile to a double ionization equation (see Equation 1) using non-linear regression analysis, the values of pKₐ of these groups were estimated to be 8.16 and 8.62. The optimum pH was found to be 8.3.

The optimum concentration of Mg²⁺ in a buffer containing 100 mM NaCl was found to be between 15 and 25 mM (panel B). Although the highest “average” rate value was obtained for 20 mM Mg²⁺, there is no statistically significant difference in the values between 15 and 25 mM Mg²⁺ as indicated by the overlap in the error bars. The effect of salt on the rate of RNA hydrolysis by RNaseE was also investigated in a buffer containing 15 mM Mg²⁺, and the optimum concentration was found to be between 100 and 200 mM (panel C). Monovalent ions such as Na⁺ tend to affect the rate of enzyme-catalyzed reactions by serving as bulk electrolytes that interact with charged surface residues rather than serving specific structural or catalytic roles, whereas divalent ions can serve both types of role. The role of Mg²⁺ divalent metals ions in the reaction catalyzed by RNaseE is being investigated further and will be reported elsewhere.

Measurement of Michaelis-Menten Parameters—We next de-

![Fig. 2. Separation and quantitation of the products of hydrolysis of BR10 by RNaseE. A, elution profiles of hydrolysis products of BR10. The RNaseE hydrolysis reaction mixture contained 400 nM substrate and 1 nM enzyme and was incubated for 30 min as described under “Experimental Procedures.” The 1-nucleotide ladder was generated by incubating 1 µg of substrate and 1 unit of S1 nuclease (Invitrogen) at 37 °C for 1 min. The positions of the p-BR10-Fl substrate and the p-BR5-Fl hydrolysis product are indicated by single and double asterisks, respectively. Numbers above each peak indicate the size of the 3’-labeled products. B, calibration of the fluorescence response. Different amounts of p-BR5-Fl from 40 fmol to 10 pmol were loaded onto the column, and the area under the corresponding peaks of fluorescence was calculated. Each value represents the average of three independent measurements. The bars represent the mean ± S.D. of each value. The inset shows the values for peak areas corresponding to amounts lower than 1 pmol of p-BR5-Fl.

![Fig. 3. The effect of pH, magnesium, and salt concentration on the rate of RNA hydrolysis catalyzed by RNaseE. The reaction buffer composition and buffering agents used to produce the required pH values are described under “Experimental Procedures.” Each kₐₜₐ₅ value is the average of three independent measurements made using the same batch of purified enzyme. Bars indicate the mean ± S.D. in each A, the effect of pH. The solid line represents the best fit of the data to the double ionization equation (see Equation 1). This yielded values of pKₐ of the enzyme-substrate complex of 8.16 and 8.62. B, the effect of magnesium concentration. The reaction buffer used for this experiment had the optimum pH of 8.3. C, the effect of salt concentration. The reaction buffer used for this experiment had a pH of 8.3 and a magnesium concentration of 15 mM. The lines in panels B and C were produced by drawing a smooth curve through the points.](image-url)
of to the Michaelis-Menten equation (Equation 2). The calculated values are less susceptible to hydrolysis (36). The within a distance of 8 nucleotides from a monophosphate group using an oligoadenosine substrate that phosphodiester bonds experiment was to further investigate an observation made hydrolysis of p-BR10-Fl (17). The reaction buffer used for this experiment had a pH of 8.3 and contained 15 mM MgCl₂ and 100 mM NaCl. The concentration of RNaseE in each reaction was 1 nM. The initial rate of each reaction was determined as described under "Experimental Procedures." Each V₀/[E] value is the average of three independent measurements made using the same batch of purified enzyme. Bars indicate the mean ± S.D. in each. The lines represent the best fit of data to the Michaelis-Menten equation (Equation 2). The calculated values of K_M and k_cat for BR13 (panel A) are 2.07 µM and 1.4 s⁻¹, and the calculated values for BR10 (panel B) are 9.70 µM and 1.1 s⁻¹, respectively.

Table I. The effects of nucleotide modifications on hydrolysis by RNaseE

| Substrate (modification) | Reaction rate constant |
|--------------------------|------------------------|
| BR10                    | (1.50 × 10⁶)          |
| BR13                    | (3.49 × 10⁷)          |
| BR10 G4(A)              | (1.25 × 10⁷)          |
| BR10 G4 (inosine)       | (3.18 × 10⁷)          |
| BR10 G4(2-amino purine) | (1.46 × 10⁷)          |
| BR10 G4(7-deaza G)      | (2.51 × 10⁷)          |
| BR10 U5(2'-OMe U)       | ND                      |
| BR10 U5(2'-NH₂ U)       | (2.97 × 10⁶)          |
| BR10 U5(2'-F U)         | (5.71 × 10⁶)          |

ND, indicates no cleavage detected at the −5 position. *, indicates low rate of hydrolysis (<1 × 10⁵ M⁻¹ s⁻¹) at the −6 position, whereas **, indicates low rate of hydrolysis at the −7 position.

Figure 4. Michaelis-Menten parameters for the hydrolysis of oligonucleotide substrates. The reaction buffer used for this experiment had a pH of 8.3 and contained 15 mM MgCl₂ and 100 mM NaCl. The concentration of RNaseE in each reaction was 1 nM. The initial rate of each reaction was determined as described under "Experimental Procedures." Each V₀/[E] value is the average of three independent measurements made using the same batch of purified enzyme. Bars indicate the mean ± S.D. in each. The lines represent the best fit of data to the Michaelis-Menten equation (Equation 2). The calculated values of K_M and k_cat for BR13 (panel A) are 2.07 µM and 1.4 s⁻¹, and the calculated values for BR10 (panel B) are 9.70 µM and 1.1 s⁻¹, respectively.

Identification of Important Functional Groups in the G De-terminant—To identify functional groups that are important for the hydrolysis of RNA by RNaseE, we used pseudo-first order conditions where K_M > [E] > [S] and consequently the normalized rate of reaction is equal to the catalytic efficiency (k_cat/K_M). The p-BR10-Fl substrate was chosen as the background to compare the effects of functional group modifications as the lower reaction rate made easier the manual collection of multiple time points. Estimates of the reaction rates for each modified p-BR10-Fl substrates (Table I) were obtained by fitting the amount of product formed against time to the pseudo-first order equation (see Equation 3). As reported previously for the two major sites of RNaseE hydrolysis in 9 S RNA (17), we found that the G one-nucleotide upstream of the susceptible phosphodiester bond in the pBR322 site is an important sequence determinant. The replacement of the G at position 4 with an A reduced the reaction rate by 12.0-fold. Compared with guanosine (Fig. 5), adenosine has an amino group in place of a keto group at the 6-position and lacks a proton at the N1-position and an amino group at the 2-position. To determine specifically whether the 2-amino group is important for hydrolysis by RNaseE, we next incorporated inosine at nucleotide 4. The reaction rate was reduced by only 4.7-fold, suggesting that a group(s) in addition to the 2-amino group has a role. This was confirmed by assaying the hydrolysis of an oligonucleotide substituted at nucleotide 4 with 2-aminopurine, which lacks the N1-proton and 6-keto group. The rate of hydrolysis of this substrate was reduced by 10.3-fold, indicating that in a guanosine upstream of a susceptible bond, the N1-proton or 6-keto group or both along with the 2-amino group contributes to the hydrolysis of RNA by RNaseE. We also found that the reaction rate of an oligonucleotide substituted with 7-deaza-G, which has a carbon in place of a nitrogen at the 7-position in the nucleobase, was reduced by 6.0-fold. The requirement for a particular group on the imidazole ring of guanosine provides an explanation for the finding that oligonucleotides substituted with uracil, which lacks an imidazole ring, in place of guanosine are cleaved by RNaseE at a reduced rate (17). The lack of a 2-amino group on uracil may also contribute to the greatly reduced rates of hydrolysis that have been observed.

The Importance of Being Ribose—Hydrolysis of p-BR10-Fl substituted with 2'-deoxyuridine at nucleotide position 5 was not detected between positions 5 and 6, indicating that the 2'-OH in nucleotide 5 is required for hydrolysis by RNaseE. One possible role for the 2'-hydroxyl group in the upstream nucleotide was that it participated in the hydrolysis reaction by forming a hydrogen bond to the 3'-leaving group oxygen. To investigate this possibility, we replaced the hydroxyl at the 2' position in nucleotide 5 with fluorine, which is unable to share a proton. This caused only a 2.6-fold reduction in the reaction
rate (Table I), indicating that proton sharing by the 2′-OH group is not critical for the RNaseE-catalyzed reaction, although it may make a modest contribution. As the sugars of 2′-fluorine-substituted nucleosides and ribonucleosides have a preference for the C3′-endo conformation whereas 2′-deoxyribonucleosides are predominantly in the C2′-endo sugar conformation (40), we next investigated whether the pucker of the sugar in the upstream nucleotide is important (Table I). This was done by synthesizing an oligonucleotide substituted at nucleotide position 5 with 2′-aminomethyl-2′-deoxyuridine, which has a preference for the C2′-endo sugar conformation (41) and is able to share a proton at the 2′ position. Hydrolysis of this oligonucleotide was barely detected. The reaction rate was reduced around 50-fold. Combined, the above results indicated that the efficient hydrolysis of RNA by RNaseE is dependent on the pucker of the ribose sugar.

Hydrolysis of an oligonucleotide substituted at nucleotide 5 with 2′-O-methyl, which blocks proton sharing while maintaining a ribose-like sugar pucker, was not detected. In light of the result with the deoxy-2′-fluorouridine-substituted substrate, this finding suggests that RNaseE hydrolysis is hampered by a bulky group at this particular position. Although 2′-O-methyl-containing oligonucleotides have been shown previously not to be cleaved by RNaseE, the cause could not be linked to a specific position because more than one substitution had been incorporated (42).

DISCUSSION

A number of factors have been shown to influence hydrolysis of RNA by E. coli RNaseE such as the sequence of hydrolysis sites, the secondary structure and 5′ phosphorylation status of the substrate, and domains within the C-terminal half of the enzyme. However, there is as yet no detailed understanding of the relative influence of these factors and how they combine mechanistically to regulate the recognition and hydrolysis of RNA by RNaseE. Although numerous RNaseE polypeptides and RNA substrates have been constructed that differ in domain composition and structure, respectively, many if not most comparisons of RNA hydrolysis in vitro have been based on limited kinetic data under conditions that have not been rigorously defined. Although this has been sufficient to identify factors that influence hydrolysis, it has not been possible to thoroughly test the correctness of models that have been proposed. A significant barrier to the generation of sufficient kinetic data to do this, at least in our case, was the labor and time required to prepare and purify radiolabeled substrates, run polyacrylamide gels, and measure band intensities from autoradiographs or phosphorimages. The new assay approach described here provides much higher throughput while being sensitive and quantitative.

The reactions were done as described previously (29) with samples being withdrawn at different time points and quenched. However, ion-pair reverse phase chromatography was used in place of polyacrylamide gel electrophoresis to separate substrate and products and an automated injection system was used to load samples. The resolution provided by ion-pair reverse phase chromatography is more than comparable with that of polyacrylamide gel electrophoresis (Fig. 2) and has also been used, for example, for footprinting studies (43). The quantity of each RNA species as it was eluted was determined by an in-line instrument that measured the amount of fluorescence from fluorescein that was attached to substrates during their chemical synthesis. The fluorescent label was placed at the 3′ end to allow RNaseE to sense the phosphorylation status at the 5′ end of substrates. This positioning also blocks 3′-exonucleolytic attack, and we have been able for the first time to use small defined substrates to assay RNaseE activity within the degradosome and crude cell extracts.5

The first polypeptide of RNaseE we have chosen to study is the N-terminal catalytic half of RNaseE. In doing so, we aim to provide a platform for investigating in future studies the role of ancillary domains within the C-terminal half of the E. coli enzyme and a reference for the many RNaseE homologues in eubacteria and plant plastids that only have sequence similarities to the NTH of E. coli RNaseE. As in previous studies (10, 17, 29, 36), we expressed NTH-RNaseE using the pET system (31–33), which generates an oligohistidine tag that facilitates purification by immobilized metal affinity chromatography (44, 45). In this study, however, we used a polypeptide that was tagged at the C rather than the N terminus, which may reduce the activity of RNaseE. In experiments not described here, we found that the amount of RNaseE produced by the pET system is inversely related to the activity of the cloned RNaseE gene and that the levels of NTH-RNaseE produced were higher when the oligohistidine tag was positioned at the N terminus. Consistent with our notion that the activity of RNaseE is affected by extension of the N terminus, there is evidence that the ability of E. coli RNaseE, an orthologue of RNaseE, when overproduced to compensate for the loss of RNaseE is affected by extension of its N terminus (compare Refs. 46 and 47). However, altering the position of the tag does not appear to affect the specificity of the enzyme (compare Fig. 2 with Ref. 29).

The assay was used first to optimize the reaction conditions for the N-terminal domain of RNaseE (Fig. 3). The substrate for these experiments was BR13, which has three guanosines followed by the sequence of the RNaseE site at the 5′ end of pBR322 RNAI (Fig. 1). Previous studies have shown that this substrate is sufficient to direct hydrolysis by purified RNaseE of the phosphodiester bond that appears in the context of RNAI to be the most susceptible to hydrolysis in vivo. A substrate

5. L. Tong and K. J. McDowall, unpublished data.
concentration of 6 µM was used. As a $K_M$ value of 0.7 µM was obtained in initial experiments (data not shown) using the starting conditions (36), the concentration of substrate was sufficiently higher than the $K_M$ at the start of optimization for the reaction rates to approach their maximum. We found that catalysis is dependent on unprotonated and protonated functional groups (panel A). The values of $pK_a$ were estimated to be 8.16 and 8.62. The environment of an amino acid side chain within a protein has been shown to affect the value of $pK_a$ by as much as 3 pH units (e.g. Refs. 35 and 48). By taking this into consideration, comparisons with the values of $pK_a$ calculated for free amino acids (49) have been used to implicate particular side chains in the reactions of certain enzymes (50, 51). In the case of RnaseE, the ionizable residues required for catalysis could correspond to lysine, tyrosine, cysteine, or histidine residues, which in the free form have $pK_a$ values of 10.4, 9.6, 8.3, and 6.3, respectively. There are a number of lysine, tyrosine, cysteine, and histidine residues within the NTH of $E. coli$ RnaseE. Of these, three lysines at positions 103, 109, and 149 and three tyrosines at positions 42, 123, and 256 are highly conserved across the RnaseE family6 and located within the smallest RnaseE polypeptide that has been found to have hydrolytic activity (52). Hydrated metal ions such as Mg(H$_2$O)$_6^{2+}$ could also be one of the ionizable groups that are required for the hydrolysis of RNA by RnaseE. Irrespective of the actual identity of the proton donor and acceptor, their detection is suggestive of general acid-general base catalysis, which is used for example by the RnaseH1 family of ribonucleases (53).

By increasing the pH from 7.6 to 8.3 and the MgCl$_2$ concentration from 10 to 15 mM (Fig. 3, panels A and B), we estimate that we have been able to increase the rate of RNA hydrolysis by at least 4-fold. This means that previous biochemical studies that investigated the relative roles of ribonucleases in various steps in RNA decay and processing, e.g. poly(A) removal (36), may have underestimated the capabilities of RnaseE. After optimizing conditions for the hydrolysis of RNA by the N-terminal half of RnaseE, we determined Michaelis-Menten parameters for the hydrolysis of p-BR13-Fi and p-BR10-Fi (Fig. 4). The latter lacks the three extra Gs at the 5′ end of BR13. The values of $k_{cat}$, we determined (1.4 and 1.1 s$^{-1}$, respectively) are similar to those of other phosphodiesterases, RnaseH II (1.5 s$^{-1}$, see Ref. 39) and EcoRV (0.70 s$^{-1}$, see Refs. 37 and 38), and may reflect similarities in the chemistries used to cleave phosphodiester bonds following the formation of an enzyme-substrate complex. The $K_M$ values calculated for p-BR13-Fi and p-BR10-Fi using the optimized conditions were 2.07 and 9.70 µM, respectively. Interestingly, the $K_M$ value calculated for p-BR13-Fi using the optimized conditions appears to be 2–3-fold higher than that calculated using the previous reaction conditions (36). This possible effect on $K_M$ is being investigated further by studying the effect of pH and magnesium on the hydrolysis of RNA under pseudo-first-order conditions as well as steady-state conditions. The higher $K_M$ value for p-BR10-Fi is consistent with the finding that phosphodiester bonds <8 nucleotides from a monophosphate group at the 5′ end are less susceptible to hydrolysis (36). By now determining kinetic parameters for reactions between RnaseE polypeptides that contain additional domains and substrates that are more complex in structure, it will be possible to address the correctness of models that have been proposed for RnaseE, such as a role for the arginine-rich RNA domain in the recruitment of structured RnaseE to the catalytic site (17) and the effect of distance from the 5′ end (36).

While we were undertaking our kinetic analysis of the N-terminal half of RnaseE, collaborators were successful in obtaining crystals of this part of RnaseE.6 We therefore decided to generate biochemical data that in hand with a 3-dimensional structure should lead to the identification of enzyme-substrate interactions that are candidates for having important roles in the hydrolysis of RNA. Our approach was to modify the RNA because, unlike the amino acid residues involved in catalysis, the targets of catalysis, i.e. susceptible bonds, had been identified. Previous studies had shown that a guanosine positioned within 1–2 nucleotides upstream of susceptible bonds is an important determinant of hydrolysis by $E. coli$ RnaseE. Therefore, we synthesized a number of oligonucleotide substrates that had functional groups substituted in the guanosine at nucleotide 4 in p-BR10-Fi. We found under pseudo-first-order conditions that at least three functional groups in this sequence determinant are required for efficient RnaseE hydrolysis of RNA (Table I). The lack of the 2-amino group along with the lack of the N1 proton or 6-keto group is sufficient to explain the reduced rate of hydrolysis of oligonucleotides containing G to A substitutions, whereas the lack of the imidazole ring along with the lack of the 2-amino group can explain the reduced rates resulting from G to U substitutions (17). The functional groups in additional sequence determinants that have recently been shown to affect the efficiency of cleavage by $E. coli$ RnaseE (54) can be characterized using the approach described here. It is now clearly established experimentally that RnaseE is sensitive to the presence of specific nucleotides at multiple positions relative to the site of cleavage even though it can hydrolyze substrates with low sequence complexity such as oligomeric adenosine and uracil (36, 55). In addition to sequence, our analysis of substitutions of the 2′-OH on the ribose sugar directly 5′ to the phosphodiester bond that is normally cleaved by RnaseE suggests that a ribose-like sugar pucker is important for efficient hydrolysis.

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