Cleavage of Oligoribonucleotides by the 2’,5’-Oligoadenylate-dependent Ribonuclease L*

(Received for publication, October 4, 1995)

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RNase L, the 2’,5’ oligoadenylate-dependent ribonuclease, is one of the enzyme systems important in the cellular response to interferon. When activated in the presence of 2’,5’-linked oligoadenylates, RNase L can catalyze the cleavage of synthetic oligoribonucleotides that contain dyad sequences of the forms UU, UA, AU, and AG, but it cannot catalyze the cleavage of an oligoribonucleotide containing only cytosines. The primary site of the cleavage reaction with the substrate C11UUUC7 has been defined to be 3’ of the UU dyad by labeling either the 5’ or the 3’ end of the oligoribonucleotide and by examining the reaction products on polyacrylamide sequencing gels. Reaction time courses have been used to determine the kinetic parameters of the cleavage reaction. The effect of the overall length of the oligoribonucleotide as well as the sequence of the bases around the position of the cleavage site on the kinetics of the cleavage reaction has been examined. The efficiency with which activated RNase L catalyzes the cleavage of the substrate C11UUUC7 is 1.9 × 107 M⁻¹ s⁻¹. Because the cleavage of the synthetic oligoribonucleotide can be used to monitor the steady-state kinetics of catalysis by activated RNase L, this method offers an advantage over previous methods of assay for RNase L activity.

One of the enzyme systems whose activity is enhanced on treatment with interferon is RNase L, the 2’,5’ oligoadenylate-dependent ribonuclease (Kerr and Brown, 1978). Interferons induce the expression of 2’,5’ oligoadenylate synthetases, which catalyze the formation of oligomers of adenosine linked 2’ to 5’ (Hovanessian et al., 1977). These 2’,5’ oligoadenylates bind to and activate the latent RNase L to cleave viral and cellular RNAs at the 3’ side of UpNp sequences (Floyd-Smith et al., 1981; Wreschner et al., 1981), leading to the inhibition of protein synthesis.

RNase L activity has been shown to be important in the mechanisms of cellular antiviral defense. Overexpression of 2’,5’ oligoadenylate synthetase leads to the inhibition of picornavirus replication (Chebath et al., 1987; Rysiecki et al., 1989). Introduction of an inactive mutant of RNase L caused an increased susceptibility to infection by picornavirus and a loss of the inhibition of cell growth caused by interferon treatment (Hassel et al., 1993). Introduction of 2’,5’ oligoadenylate into cells (Hovanessian and Wood, 1980) or the expression of 2’,5’ oligoadenylate synthetase (Rysiecki et al., 1989) have been found to cause growth arrest, suggesting a role for the RNase L system in the regulation of cell growth.

The genes for human and murine RNase L have been cloned and sequenced (Zhou et al., 1993). Overexpression of RNase L employing baculoviral vectors and insect cell lines (Dong et al., 1994) has supplied enough material for protein chemical experiments to be performed. The recent demonstration of the formation of dimers of RNase L in the presence of activators (Dong and Silverman, 1995) has opened up new areas of investigation regarding the mechanism of action of the activated complex and has created the need for detailed investigation of the kinetics of catalysis by RNase L.

Current methods of assay for RNase L activity rely on the cleavage of radiolabeled poly(U) (Silverman, 1985) followed by quantification of the remaining substrate by precipitation or on the cleavage of ribosomal RNA (Karako et al., 1987) followed by analysis of specific cleavage products with agarose gel electrophoresis. These methods are useful for the detection of RNase L activity, but detailed examination of the steady-state kinetics of catalysis by RNase L requires a quantitative method. This work examines the cleavage by activated RNase L of synthetic oligoribonucleotides containing one or two sites of cleavage as a means of quantitatively determining the steady-state activity of the enzyme. The effect of overall length of the oligoribonucleotide and the sequence of bases at the site of cleavage on the catalytic efficiency and position of cleavage are examined.

MATERIALS AND METHODS

Enzyme—The gene encoding RNase L was cloned from a human kidney cDNA library (Clontech) using polymerase chain reaction and the following primers: 5’-GAATTCGGATCACAAGCTTCATATGGGAAGCAGGGATCATAACAACCCC-3’ and 5’-GAATTCGGATCCAAAGCTTTCAGCACCCAGGGCTGGCCAACCCACT-3’. The sequences of the oligonucleotides used for polymerase chain reaction were based on the published sequence of RNase L (Zhou et al., 1993). The polymerase chain reaction-deduced RNase L gene was completely sequenced using dideoxy methodology, which confirmed the published sequence with the exception of one amino acid. The deposited sequence (GenBank accession number L10381) has been corrected.

RNase L was overproduced in insect cell line TenS1-4 using a baculoviral vector. Recombinant virus was produced using the Baculogold system (Pharmingen, San Diego, CA) according to the supplier’s instructions. TenS1-4 cells in spinner flasks in Ex-Cell 401 medium (J RH Bioscience, Lenexa, KS) at 1–2 × 10⁶ cells/ml at 27°C were infected at a multiplicity of infection of 5 and were harvested 72 h post-infection. The purification of RNase L was accomplished according to published procedures (Dong et al., 1994) with the following exception. Instead of chromatography on Superose 12, the protein was chromatographed on Biospin-6 columns (Bio-Rad) that had been equilibrated with 25 mM Tris, pH 7.5, 100 mM KCl, and 5.8 mM magnesium acetate. Approximately 4 mg of RNase L were purified from 3.7 × 10⁸ cells. The RNase L was 90% pure as judged with SDS-polyacrylamide gels and Coomassie Blue staining.

Protein concentrations were determined using amino acid analysis. Attempts to sequence the RNase L with automated Edman degradation were unsuccessful, suggesting that the N terminus of the enzyme was blocked. Sequencing of peptides generated by trypsin-catalyzed hydrolysis confirmed the presence of the published amino acid sequence.
Cleavage of Oligoribonucleotides by RNase L

Purified RNase L was stored in 25 mM Tris, 100 mM KCl, 5 mM magnesium acetate, pH 7.5, and 50% glycerol at −70 °C.

RNA Synthesis—Oligoribonucleotide substrates and 2′,5′-linked oligoadenylate activators were synthesized by Midland Certified Reagent Company (Midland, TX). Because of the high content of cytosines of the substrates, a 30-h deprotection in tetrahydroin ammonium fluoride was required. RNA substrates were purified by electrophoresis on 20% polyacrylamide, 7 M urea/TBE (90 mM Tris borate and 1 mM EDTA) sequencing gels. Bands were located with UV shadowing and excised from the gel with a scalpel. The gel pieces were crushed with a stirring rod, and the oligoribonucleotide was eluted by soaking in 300 mM sodium acetate, pH 5. The oligoribonucleotide was then precipitated with the addition of 5 volumes of ethanol, centrifuged, and resuspended in 10 mM HEPES, pH 7.0. The C,S oligoribonucleotide was purified with C-18 Sep-pak cartridges (Waters) because the oligoribonucleotide remained soluble in ethanol. Concentrations of oligoribonucleotides were determined from absorbance spectra using molar extinction coefficients calculated from the sequences. The activator used in the present study, 5′-monophosphate 2′,5′-adenosine trimer (p2,5An), was purified using high pressure liquid chromatography on a C-18 column.

Oligoribonucleotide substrates were radiolabeled at the 5′ position with [α-32P]-ATP (NEN, 6000 Ci/mmol) and polynucleotide kinase (U. S. Biochemical Corp.). 5′-end-labeled oligoribonucleotides were purified from the kinetic reaction with ethanol precipitation. Radiolabeling at the 3′ position of oligoribonucleotides was carried out using 5′-[32P]-3′-cytidine bisphosphate (NEN, 3000 Ci/mmol) and RNA ligase (New England Biolabs), according to the supplier’s protocol, followed by ethanol precipitation.

Steady-state Kinetics of Cleavage—Reactions contained 11.5 mM HEPES, 104 mM KCl, 5.8 mM magnesium acetate, 5 mM dithiothreitol, 50–2000 nM oligoribonucleotide, 0.2% polyethylene glycol 8000, 1.2 mM ATP, 50–800 nM p2,5A5, and 0–600 nM RNase L in a total volume of 50 μl at a pH of 7.6 and at 30 °C. To avoid the loss of enzyme activity due to protein adsorption to the surface of the reaction tube, reactions were carried out in polypropylene microcentrifuge tubes that had been incubated for several hours with 1% polyethylene glycol 20,000 in RNase-free H2O and then blown dry and incubated at 65 °C for 30 min. RNase L was preactivated by incubating enzyme (300 pM to 2.5 nM) and 500 or 800 nM substrate C11UUC7 against synthetic oligoribonucleotide markers and against the products resulting from the cleavage of 5′-[32P]-3′-fluoroU)UC7 creates a ladder of 3′-phosphate-terminated oligos (Fig. 1, lane 1). The lengths of the 5′-phosphate-terminated oligos (Fig. 1, lane 1). The lengths of the 5′-phosphate-terminated oligos (Fig. 1, lane 1). The lengths of the 5′-phosphate-terminated oligos (Fig. 1, lane 1). Therefore, the product band was a fraction of the total of the counts in the substrate and product bands and multiplying by the initial substrate concentration, thus eliminating errors due to loading different volumes of the samples on the gel. Cleavage of substrate C11UUC7, by RNase Pho M (Sigma) was carried out according to the supplier’s instructions using 3 units of enzyme in a 30-μl reaction. Base-catalyzed hydrolysis of oligoribonucleotides was accomplished by incubating the 5′-labeled oligoribonucleotide in 100 mM Na2CO3, pH 9, at 85 °C for 1 h.

Data Analysis—All nonlinear regression calculations were performed as described previously (Carroll et al., 1993). Rate saturation data were fit directly to the Michaelis-Menten equation to determine Kₘ and kₐ. The values for kₐ reported in Table I were calculated based on the concentrations of monomer in the reactions, assuming that dimer formation was completed during the preincubation with activator.

**RESULTS**

Cleavage of Oligoribonucleotides by RNase L—The sequence of the substrate C11UUC7 was based on studies of the cleavage of viral mRNAs by RNase L that demonstrated a preference for cleavage afterUU and UA sequences (Wreschner et al., 1981).

![Image](http://www.jbc.org/)

**Table I**

| Substrate       | kₐ (μM⁻¹s⁻¹) | kₘ (μM⁻¹) | kₐ/kₘ (μM⁻¹s⁻¹) |
|-----------------|--------------|-----------|-----------------|
| C11UUC7         | 3.4          | 180       | 1.9 × 10⁴       |
| C11UUC₄         | 7.9          | 1400      | 5.6 × 10⁴       |
| C11UAC₃         | 3.7          | 110       | 3.4 × 10⁴       |
| C11UUC₆         | 7.6          | 150       | 5.0 × 10⁴       |
| C11UC₅          | 0.54         | 670       | 8.0 × 10⁴       |
| C11UC₇          | 1.0          | 160       | 6.1 × 10⁴       |
| C11UGC₇         | 1.9          | 140       | 1.4 × 10⁴       |
| C11AUC₇         | 0.7          | 500       | 1.4 × 10⁴       |
| C11AUC₉         | 1.1          | 175       | 6.2 × 10⁴       |

Floyd-Smith et al., 1981). For the present study the sequence flanking the cleavage dyad included only cytosines to ensure the lack of self-complementarity. The addition of activator p2,5A₅ to a reaction containing 5′-[32P]-C11UUC₇ and RNase L led to the formation of one major radiolabeled product band that migrated on a 20% acrylamide/7 M urea/TBE gel at approximately the position of a 12-mer (Fig. 1, lane 7). In reaction time courses, the intensity of the Phosphor image of the radiolabeled product band increased with longer reaction time. In the absence of either activator or RNase L, no product band was detected (Fig. 1, lanes 5 and 6, respectively). The addition of a higher concentration of RNase L or an increase in the reaction time resulted in the appearance of another product band migrating one nucleotide shorter on the gel, after almost all of the substrate had first been converted to the initial product (Fig. 1, lane 8).

**Site of Cleavage**—Comparison of the position on the gel of the product band corresponding to the first cleavage reaction with substrate C11UUC₇ against synthetic oligoribonucleotide markers and against the products resulting from the cleavage of 5′-[32P]-C11UUC₇ with RNase Phy M is also shown in Fig. 1. RNase Phy M catalyzes the cleavage of RNA preferentially to the 3′ side of U and A generating RNA products with a terminal 3′ phosphate (Donis-Keller, 1980). Activated RNase L generates on cleavage of C11UUC₇, a major product that migrates to the same position as the larger of the two major products generated by RNase Phy M (Fig. 1, lanes 8 and 9). Because this product migrates between 5′-[32P]-C11U (Fig. 1, lane 10) and 5′-[32P]-C₅, U (Fig. 1, lane 11), it most likely corresponds to 5′-[32P]-C₅, U with a 3′ phosphate.

Further evidence to pinpoint the site of cleavage of C11UUC₇ comes from comparison of base-catalyzed hydrolysis of 5′-3-phosphorylated oligoribonucleotides. Base-catalyzed hydrolysis of 5′-3-phosphorus-C₅, UUC₇, and of 5′-3-phosphorus-C₅, (2′-fluoro)UUC₇ creates a ladder of 3′ phosphate-terminated oligos (Fig. 1, lanes 1 and 2). The hydrolysis product that is present in the base-catalyzed hydrolysis of C11UUC₇, but is absent in the hydrolysis of C11(2′-fluoro)UUC₇, corresponds to C11(U₃)-3′. Because the major cleavage product of C11UUC₇ by RNase L is one nucleotide longer than this position, the product corresponds to C11U(U₃)-3′.

That a single cleavage event leads to the production of 5′-end-labeled C11,UUP is demonstrated by examination of the reaction products from RNase L-catalyzed cleavage of 3′-end-labeled C11UUC₇ cytidine 3′,5′-bisphosphate (Fig. 1, lane 14). A single radiolabeled product is formed that migrates to the same position as the smaller of the two products generated by cleavage of C11UUC₇, cytidine 3′,5′-bisphosphate by RNase Phy M (Fig. 1, lane 15). This radiolabeled product migrates with the same mobility as authentic C₇ cytidine 3′,5′-bisphosphate produced by RNA ligase-catalyzed ligation of C₇ and cytidine 3′,5′-bisphosphate (Fig. 1, lane 13).

The lengths of the 5′ cleavage products of the other sub-
substrates used in this study are determined by comparison with the product from the cleavage of C11UUC7 and are shown in schematic form in Fig. 2. Substrates containing the dyad sequence UU are cleaved at the same positions relative to the UU dyad. The addition of a third U, as in substrate C11UUUC7, does not change the primary cleavage site. Activated RNase L catalyzes the cleavage of C11AAC7 and C11UUC7 between the nucleotides of the dyads. Activated RNase L catalyzes the cleavage of C11UC8 and C11UGC7 in two positions.

Initial Velocity—A lag in product formation is observed in reaction time courses that are initiated by the addition of RNase L to a solution containing activator, p2,5A3, and substrate, as shown in Fig. 3. Reaction time courses that show a linear increase in product with increasing reaction time are obtained in reactions that are initiated by the addition of RNase L that is preactivated by incubation in the presence of p2,5A3. Control experiments demonstrate that preincubation of 2.5 nM RNase L with p2,5A3 (800 nM) on ice for 30 min in reaction buffer (11.5 mM HEPES, pH 7.5, 104 mM KCl, 5.8 mM MgOAc2, 5 mM dithiothreitol) containing 0.2% polyethylene glycol 8000 result in the maximal rate of reaction. Further control experiments show that enzyme activity in the activated stock solution is stable for at least 1 h when stored on ice (data not shown).

Steady-state Kinetics—The rate of cleavage of C11UUC7 as a function of the concentration of enzyme is shown in Fig. 4. For this experiment the enzyme was preactivated by preincubation at 2.5 nM in the presence of 800 nM p2,5A3. The plot of the rate of cleavage of substrate, C11UUC7, as a function of the concentration of preactivated RNase L in the reaction, is linear over the enzyme concentration range tested (100–600 pM). The rates of the individual reactions are linear, indicating that the enzymatic activity in the reaction is constant over the reaction time observed (2.5 min).

As shown in Fig. 5, the rate of cleavage of C11UUC7 increases with increasing concentrations of p2,5A3, and approaches a maximal rate at about 10 nM with half-maximal activation at a concentration of p2,5A3 of 1 nM. For this experiment reactions were initiated by the addition of RNase L that had been incubated with the same concentration of p2,5A3 that was included in the subsequent cleavage reaction. Reaction time courses were monitored for product formation at 2.5 min. Higher concentrations of p2,5A3, up to 1 μM, gave the same maximal reaction rate.

Substrate Specificity—The steady-state kinetic parameters for cleavage of several substrate oligoribonucleotides as catalyzed by RNase L studied. The arrows indicate the primary site(s) of cleavage.
Cleavage of Oligoribonucleotides by RNase L

Effect of the RNA Sequence on the Rate of Cleavage—For substrates that are cleaved at more than one position, all of the radioactive reaction products have been added together to determine the total rate of cleavage of the substrate. RNase L catalyzes the cleavage of oligoribonucleotides containing either 2 or 3 sequential Us with efficiencies of cleavage that are approximately 20- and 50-fold higher, respectively, than the efficiency with which the enzyme cleaves a single U. The increased efficiencies of cleavage are due to increases in \( k_{\text{cat}} \) and decreases in \( K_m \).

Activated RNase L cleaves substrates containing a single U and 2 or 3 sequential Us more efficiently than the substrate with the single U. A substrate with three consecutive Us is cleaved 2.3 times more efficiently than the substrate with the single U. The increases in the efficiency of cleavage are primarily due to increases in \( k_{\text{cat}}/K_m \).

In vivo, secondary structural characteristics associated with individual RNA sequences may mask potential cleavage sites or possibly enhance the efficiency of cleavage of specific sites. However, the value of \( k_{\text{cat}} \) (7.6 s\(^{-1}\); see Table I) for the cleavage of \( C_{11}UUUC_7 \) is comparable with the rate of cleavage of a
specific mRNA catalyzed by RNase L that has been activated by a 2',5' adenosine oligomeric DNA chimera (7 s⁻¹; Maitra et al., 1995).

Recent investigations of the quaternary structure of the activated RNase L have indicated that the enzyme is dimerized in the presence of activator (Dong and Silverman, 1995; Cole et al., 1996). The existence of a quantitative assay for RNase L activity will make possible the determination of the dynamic relationship between the oligomeric/liganded states of the activated complex and its catalytic activity.

REFERENCES

Carroll, S. S., Olsen, D. B., Bennett, C. D., Gotlib, L., Graham, D. J., Condra, J. H., Stern, A. M., Shafer, J. A., and Kuo, L. C. (1993) J. Biol. Chem. 268, 276–281
Chebath, J., Benech, P., Revel, M., and Vigneron, M. (1987) Nature 330, 587–588
Cole, J. L., Carroll, S. S., and Kuo, L. C. (1996) J. Biol. Chem. 271, 3979–3981
Dong, B., and Silverman, R. H. (1995) J. Biol. Chem. 270, 4133–4137
Dong, B., Xu, L., Zhou, A., Hassel, B. A., Lee, X., Torrence, P. F., and Silverman, R. H. (1994) J. Biol. Chem. 269, 14153–14158
Donis-Keller, H. (1980) Nucleic Acids Res. 8, 3133–3142
Floyd-Smith, G., Slattery, E., and Lengyel, P. (1981) Science 212, 1030–1032
Hassel, B. A., Zhou, A., Sotomayor, C., Maran, A., and Silverman, R. H. (1993) EMBO J. 12, 3297–3304
Hovanessian, A. G., and Wood, J. N. (1980) Virology 101, 81–90
Hovanessian, A. G., Brown, R. E., and Kerr, I. M. (1977) Nature 268, 537–539
Kariko, K., Sobol, R. W., Suhaddonick, L., Li, S. W., Reichenbach, N. L., Suhaddonick, R. J., Charubala, R., and Pfleiderer, W. (1987) Biochemistry 26, 7127–7135
Kerr, I. M., and Brown, R. E. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 256–260
Maitra, R. K., Li, G., Xiao, W., Dong, B., Torrence, P. F., and Silverman, R. H. (1995) J. Biol. Chem. 270, 15071–15075
Rysiecki, G., Gewert, D. R., and Williams, B. R. G. (1989) J. Interferon Res. 9, 649–657
Silverman, R. H. (1985) Anal. Biochem. 144, 450–460
Wrechni, D. H., McCauley, J. W., Skehel, J. J., and Kerr, I. M. (1981) Nature 289, 414–417
Zhou, A., Hassel, B. A., and Silverman, R. H. (1993) Cell 72, 753–765
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J. Biol. Chem. 1996, 271:4988-4992.
doi: 10.1074/jbc.271.9.4988

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