Characteristic Ribonucleolytic Activity of Human Angiogenin†

Robert Shapiro, James F. Riordan, and Bert L. Vallee*

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Angiogenin, a blood vessel inducing protein isolated from a human tumor cell line, has been found to exhibit ribonucleolytic activity. It catalyzes the cleavage of both 28S and 18S ribosomal RNA as determined by agarose gel electrophoresis. The major products formed with these substrates are 100–500 nucleotides in length. In contrast, angiogenin is inactive toward all of the more conventional substrates of the homologous pancreatic ribonucleases. In particular, it does not produce detectable amounts of acid-soluble fragments from high molecular weight wheat germ RNA, poly(C), or poly(U), nor does it hydrolyze cytidine or uridine cyclic 2′,3′-phosphate. The high degree of sequence homology between angiogenin and the pancreatic ribonucleases, which includes all three catalytic residues, His-12, Lys-41, and His-119, has thus identified the chemical nature of a potential angiogenin substrate. These results may bear importantly on the physiological function of angiogenin.

Recent reports from this laboratory have described the isolation, characterization, amino acid sequence, and gene structure of angiogenin, a blood vessel inducing protein secreted by the human colon adenocarcinoma cell line HT-29 (Fett et al., 1985; Strydom et al., 1985; Kurachi et al., 1985). The primary sequence of angiogenin is highly homologous to that of the pancreatic ribonucleases (EC 3.1.27.5) (RNases)[1] and includes their three major active site residues, His-12, Lys-41, and His-119. This suggested that angiogenin might have nucleolytic activity toward RNA though not necessarily with the same specificity as pancreatic RNase. Indeed, the initial reports noted that angiogenin is devoid of the typical activity of the pancreatic RNases.[2]

We here confirm in detail that angiogenin has no significant activity in any of the standard assays employed with pancreatic RNases. It does not catalyze the hydrolysis of C>p or U>p, nor does it produce measurable amounts of acid-soluble fragments from poly(C), poly(U), or wheat germ RNA. It also fails to act upon a wide variety of other nucleotides and polynucleotides that are not cleaved by the pancreatic enzyme. Nevertheless, the remarkable homology between these proteins compelled us to pursue the examination of ribonucleic acid(s) as possible substrates for angiogenin using high molecular weight ribosomal RNA. Indeed, we discovered endonucleolytic cleavage of both 28S and 18S RNA. In contrast with the products generated by pancreatic RNase, however, with these substrates the major products formed are much larger, i.e., 100–500 nucleotides.

Experimental Procedures

Materials. Poly(A)·poly(U), poly(I)·poly(C), poly(U)·poly(dA), poly(C)·poly(dI), poly(G)·poly(dC), and poly(A)·poly(dT) were products of P-L Biochemicals. Wheat germ RNA (high molecular weight) was from Calbiochem-Behring. Bovine pancreatic RNase A was obtained from Cooper Biomedical. GTP, GDP, 5′-GMP, guanosine cyclic 3′,5′-phosphate (cGMP), ATP, ADP, 5′-AMP, 3′-AMP, adenosine cyclic 3′,5′-phosphate (cAMP), and all 2′,5′-dinucleotides were purchased from Sigma Chemical Co. Sources of other materials are identical with those listed in Shapiro et al. (1986).

Purification of Angiogenin. Angiogenin was isolated from medium conditioned by cells from the human colon adenocarcinoma line HT-29 according to a modification of the procedure described by Fett et al. (1985). Alternative procedures for preparing angiogenin by recombinant DNA techniques and from different sources will be described in separate communications. Briefly, the major purification steps were acidification, CM-52 cation-exchange chromatography, Mono S cation-exchange chromatography, and Synchropak RP-P C18 reversed-phase HPLC. All procedures through the CM-52 chromatography were identical with those employed previously. Subsequent protocol revisions were made in order to obtain an angiogenin preparation that is free of all traces of a potent general RNase ("HT-RNase") which elutes several minutes before angiogenin on C18 HPLC (Shapiro et al., 1986).

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* Address correspondence to this author.

[1] Abbreviations: RNase(s), ribonuclease(s); C>p, cytidine cyclic 2′,3′-phosphate; U>p, uridine cyclic 2′,3′-phosphate; poly(C), poly(cytidylic acid); poly(U), poly(uridylic acid); poly(A), poly(adenylic acid); poly(I), poly(inosinic acid); poly(dA), poly(deoxyadenylic acid); poly(dI), poly(deoxyinosinic acid); poly(dC), poly(deoxycytidylic acid); poly(dT), poly(thymidylic acid); GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; 5′-GMP, guanosine 5′-phosphate; cGMP, guanosine cyclic 3′,5′-phosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; 5′-AMP, adenosine 5′-phosphate; 3′-AMP, adenosine 3′-phosphate; cAMP, adenosine cyclic 3′,5′-phosphate; UpN and CpN, uridine and cytidine dinucleotides where N represents A, C, G, or U; CM, carboxymethyl; C18, octadecylsilane; HPLC, high-performance liquid chromatography; HT-RNase, a general ribonuclease secreted by HT-29 cells (Shapiro et al., 1986); Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HSA, human serum albumin; TFA, trifluoroacetic acid; 3′-CMP, cytidine 3′-phosphate; 3′-UMP, uridine 3′-phosphate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; UpcA, a synthetic analogue of the dinucleotide UpA in which the 5′-oxygen atom of the ribose attached to adenine is replaced by a methylene group.

[2] The pancreatic ribonucleases (ribonucleate 3′-pyrimidinooligonucleotidohydrolase) catalyze the endonucleolytic cleavage of RNA and oligoribonucleotides to small, acid-soluble 3′-phosphomononucleotides and 3′-phosphooligonucleotides ending in Cp or Up with 2′,3′-cyclic phosphate intermediates. They also act directly on U>p and C>p.

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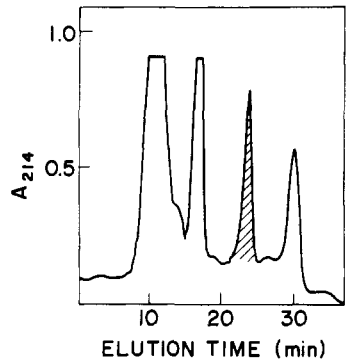

FIGURE 1: Chromatography of 11.3 mg of CM 2 fraction from acidified serum-free HT-29 cell conditioned medium on a Mono S cation-exchange column. Lyophilized CM 2 was reconstituted in 1.4 mL of 10 mM Tris, pH 8.0, containing 200 mM NaCl, and applied to the column. After 15 min of washing with the same buffer (elution profile not shown), a 50-min linear gradient from 200 to 600 mM NaCl in 10 mM Tris, pH 8.0, was applied. The flow rate was 0.8 mL/min. Column effluents were monitored by using a Waters 214-nm monitor in conjunction with a Hewlett-Packard 3390A integrator. Angiogenin (indicated by crosshatches) elutes at 22.8 min.

Material eluting from the CM-52 column in 1 M NaCl was dialyzed vs. water and lyophilized. After reconstitution in 10 mM Tris, pH 8.0, containing 200 mM NaCl, it was applied to a Mono S cation-exchange column (50 × 5 mm; Pharmacia). Angiogenin was eluted with a 50-min linear gradient from 200 to 600 mM NaCl in 10 mM Tris, pH 8.0, at a flow rate of 0.8 mL/min at room temperature (Figure 1). A Waters Associates liquid chromatography system was employed together with a Hewlett-Packard 3390A integrator. Column effluents were monitored at 214 nm and 1-min fractions were collected. Those containing angiogenin were then injected onto a Synchropak C18 column (250 × 4.1 mm; Synchrom Inc.) that had previously been equilibrated with a 4:1 mixture of 0.1% TFA (solvent A) and 2-propanol/acetonitrile/water (3:2:2 v/v/v) containing 0.08% TFA (solvent B). Angiogenin was eluted with a 90-min linear gradient from 20% to 40% B at a flow rate of 1 mL/min. One-minute fractions were collected. Angiogenin obtained by this procedure was then rechromatographed on the same C18 column, now with a 45-min linear gradient from 30% to 40% B.

Protein Assays. Concentrations of angiogenin were determined by amino acid analysis, and those of bovine RNase A were based on absorbance at 280 nm by using ϵ_{280} = 9800 M^{-1} cm^{-1} (Sela & Anfinsen, 1957).

Enzymatic Assays. Angiogenin preparations were tested for angiogenesis activity on the chick chorioallantoic membrane (Fett et al., 1985) and typically were positive at the 0.01% significance level or better.[3] Activity toward polyhomoribonucleotides was measured by a modification of the method of Zimmerman and Sandeen (1965). For the standard assay, incubation mixtures contained 40 mM Tris, pH 8.0, 100 μg of substrate, 5 μg of human serum albumin (HSA), and 20 nM angiogenin in a volume of 250 μL. Incubations were carried out at 37 °C for 2–3 h, after which time 250 μL of ice-cold 1.2 N perchloric acid containing 20 mM lanthanum nitrate was added. After 15 min on ice, the reaction mixture was centrifuged at 15600*g* for 15 min at 4 °C. The supernatant was then diluted 3-fold with water and its absorbance measured at 260 nm [poly(A), poly(G), poly(U)] or at 280 nm [poly(C)]. All readings were corrected for the absorbance of blanks that lacked angiogenin. Under these conditions, 1.6 pM bovine pancreatic RNase A produces an $A_{280} \simeq 1.0$ for poly(C), while 86 pM gives an $A_{260} \simeq 1.0$ for poly(U). In some instances alternative conditions were employed where Tris was replaced by sodium acetate, pH 5.2, or by sodium borate, pH 9.5, or the assay mixtures were supplemented as described in the text. A similar procedure was followed with poly(I)·poly(C), except that the substrate was dissolved in 0.1 M Tris, pH 7.5, containing 0.1 M NaCl and heated for 10 min at 45 °C immediately prior to use, and the final incubation mixtures contained 80 mM Tris, pH 7.5, with 80 mM NaCl. For poly(A)·poly(U), the lyophilized powder supplied by the manufacturer contained 9 parts by weight phosphate-buffered saline. It was reconstituted in water to 1 mg/mL nucleotide and heated as with poly(I)·poly(C). Incubation mixtures were prepared by combining 100 μL of substrate, 100 μL of 0.1 M Tris, pH 7.5, containing 0.1 M NaCl, 10 μL of sample, and 40 μL of 0.01% HSA. For DNA·RNA hybrids, the procedure employed was the same as that described for poly(I)·poly(C) except that the concentration of the stock substrate solution was 5 A_{260} units/mL. In this case, samples were not diluted prior to measuring absorbance.

Activity toward wheat germ RNA was measured by the production of perchloric acid soluble material according to the method of Blank and Dekker (1981). Reaction mixtures containing 35 mM Tris, pH 8.5, 50 mM sucrose, 150 mM NaCl, 0.8 mg of RNA, and 30 μg of HSA in a volume of 300 μL were incubated at 37 °C for 4.5 h. An angiogenin concentration of 28 nM was employed. The reaction was stopped by addition of 700 μL of ice-cold 3.4% perchloric acid, and after 10 min on ice the samples were centrifuged at 15600*g* for 10 min at 4 °C. The absorbance of the undiluted supernatant was measured at 260 nm. Under these conditions a bovine pancreatic RNase A concentration of 8 pM will cause an increase in A_{260} of $\simeq$1.0. In order to avoid blank values prohibitively high due to RNA degradation in the absence of added sample during these prolonged incubations, RNase contaminants in the water and buffer solutions were removed by passage through a Sep-Pak C18 cartridge (Waters).

Spectrophotometric assays of activity toward C>p and U>p were carried out by standard methods (Richards, 1955; Crook et al., 1960). Angiogenin (18–36 nM) was incubated with 0.28 mM C>p or 0.58 mM U>p in 0.1 M Mes, pH 6.5, containing 0.1 M NaCl at 37 °C, and the absorbance at 284 (C>p) or 280 nm (U>p) was continuously monitored with a Gilford Model 250 spectrophotometer. Activity toward these cyclic nucleotides was also examined by a more sensitive assay based on detection of products by HPLC (Shapiro et al., 1986). Angiogenin (28 nM) was incubated with 0.2 mM substrate in 0.1 M Mes, pH 6.0, containing 0.1 M NaCl and 10 μg/mL HSA for 12–15 h at 22 °C. An aliquot of this mixture was then injected onto a Radial-Pak C18 column (Waters). A 35-min linear gradient from 0.1 M potassium phosphate, pH 7.0, to 90 mM potassium phosphate, pH 7.0, in 32.5% (v/v) methanol was applied at a flow rate of 0.8 mL/min at room temperature. The absorbance at 254 nm was monitored with a Waters Model 440 detector and a Hewlett-Packard 3390A integrator. By this method, it is possible to measure velocities that are 10^4-fold slower than would be observed with an equivalent concentration of bovine pancreatic RNase A.

This HPLC method was also used to measure activity toward dinucleotides. Incubations were performed for 2 h at 22 °C with 7.4 nM angiogenin and 0.1 mM substrate in 0.1 M Mes, pH 6.0, containing 0.1 M NaCl. In such cases, a reaction velocity could be detected even if it was 10^5-fold slower than that observed with the same concentration of bovine

[3] Pancreatic RNase was also tested for angiogenesis activity on the chick chorioallantoic membrane but with negative results.

Table I: Potential Substrates Tested with Angiogenin[a]

Pancreatic RNase Substrates	
poly(C)	C>p
poly(U)	U>p
yeast RNA[b]	CpN[c]
wheat germ RNA	UpN
Other Nucleotides	
poly(A)	UpA(2′,5′)
poly(G)	GTP
poly(I)·poly(C)	GDP
poly(A)·poly(U)	5′-GMP
poly(A)·poly(dT)	cGMP
poly(C)·poly(dI)	ATP
poly(G)·poly(dC)	ADP
poly(U)·poly(dA)	5′-AMP
ApN	3′-AMP
GpN	cAMP
CpA(2′,5′)	3′-CMP
CpC(2′,5′)	3′-UMP

[a] Assay methods are described under Experimental Procedures. [b] Shapiro et al. (1986). [c] N = A, C, G, and U.

RNase A acting on CpA. Activities toward GTP, GDP, 5′-GMP, cGMP, ATP, ADP, 5′-AMP, 3′-AMP, cAMP, 3′-CMP, and 3′-UMP were measured by using the same method except that incubations of all but the last two compounds were performed in 0.1 M Tris, pH 7.5, containing 0.1 M NaCl, 5 mM $MgSO_4$, and 10 μg/mL HSA.

Activity toward RNA isolated from HT-29 cells was examined by agarose gel electrophoresis under denaturing conditions (Maniatis et al., 1982). RNA (15–20 μg) was incubated with angiogenin at 37 °C in either 30 mM Hepes or 30 mM Tris, pH 7.5, containing 30 mM NaCl in a total volume of 13 μL. The reaction was terminated by addition of 48 μL of a 15:5:3 mixture (v/v) of freshly deionized formamide, 37% formaldehyde, and 200 mM Mops containing 10 mM EDTA and 50 mM sodium acetate. This mixture was then heated for 10 min at 65 °C, cooled on ice, and supplemented with 6 μL of 0.25% bromophenol blue in 40% glycerol. Forty microliters of this solution was then loaded onto a 1.1% agarose gel containing 6% formaldehyde and electrophoresed at a constant voltage of 150 V. RNA was visualized with ethidium bromide.

Results

Angiogenin was first tested for activity toward a variety of pancreatic RNase substrates (Table I, top). With the polymeric substrates, standard assays were employed that measure the production of perchloric acid soluble oligonucleotides. With the dinucleotides and cyclic phosphates, activities were examined both by standard continuous spectrophotometric methods and by HPLC, the latter technique being several orders of magnitude more sensitive than the former (Shapiro et al., 1986; see Experimental Procedures). In no instance was it possible to detect any activity of angiogenin toward any of these substrates.

In view of the significant structural homology of angiogenin to the pancreatic ribonucleases (Kurachi et al., 1985; Strydom et al., 1985; see Discussion), the reaction conditions chosen for the above assays were those that are typically employed with these enzymes and, particularly, with bovine ribonuclease A. Angiogenin might, of course, require a different pH, ionic strength, additional cofactors, or a metal chelating agent in order to exhibit activity. Hence, several other assay conditions were employed to examine this possibility. Thus, the poly(U), poly(C), and wheat germ RNA assay mixtures were supplemented with either 2.5 mM magnesium and calcium; 0.1 mM zinc; 5 mM DTT; or 1 mM EDTA. Further, poly(U) assays were performed at pH 5.2 and 9.5, and under standard conditions (pH 8.0) in the presence of 0.3 M NaCl. None of these alterations resulted in detectable activity.

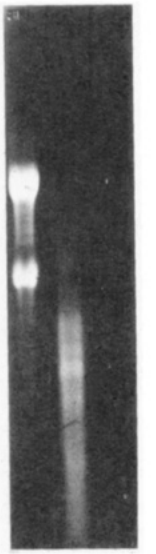

FIGURE 2: Effect of angiogenin on HT-29 cell total RNA. RNA was incubated with or without angiogenin at 37 °C as described under Experimental Procedures. After 90 min, formamide/formaldehyde reagent was added. Samples were then run on a 1.1% agarose gel under denaturing conditions. The gel was stained with ethidium bromide (0.5 μg/mL) and photographed under ultraviolet illumination. (Lane 1) Control RNA sample; (lane 2) sample containing 0.35 μM angiogenin.

These results clearly demonstrate that angiogenin does not exhibit activity characteristic of the pancreatic RNases. The possibility was therefore considered that angiogenin might have nucleolytic activity, but with an entirely different specificity. Thus, RNA·DNA hybrids, double-stranded polyribonucleotides, and 2′,5′-linked dinucleotides, among others, were examined as potential substrates (see Table I, bottom, for a complete list). Again, no significant activity was detected in any instance.

We next examined the possibility that angiogenin could be a highly specific ribonuclease that cleaves RNA at a much more limited number of sites than do the pancreatic RNases. Under such circumstances the products of endonucleolytic activity might not be acid-soluble, and hence, activity would not be detected by the usual precipitation methods employed for pancreatic RNases. Therefore, we chose an RNA preparation from HT-29 cells as a convenient substrate and monitored its degradation by agarose gel electrophoresis rather than by measuring the formation of acid-soluble products. Importantly, this RNA preparation contains essentially intact 28S and 18S rRNA, having ~5000 and ~2000 bases, respectively, which appear as distinct ethidium bromide staining bands. This enables the method to discern even a single internal cleavage in either of the RNA species.

The sample of HT-29 cell RNA (1.4 mg of RNA/mL) was incubated with 0.35 μM angiogenin at 37 °C for 90 min, and the reaction was then terminated by the addition of the formamide/formaldehyde reagent (see Experimental Procedures). As indicated in Figure 2, lane 2, agarose gel electrophoresis under denaturing conditions reveals that both the 28S and the 18S ribosomal RNA bands, so prominent in the controls (lane 1), have disappeared and polynucleotide degradation products have been formed. The association of this ribonucleolytic activity with angiogenin was examined by testing column fractions spanning the angiogenin peak obtained by C18

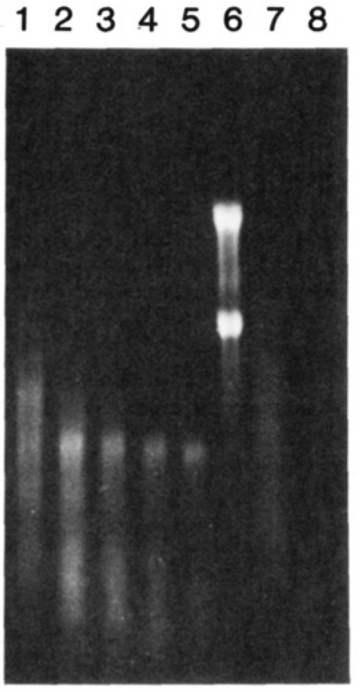

FIGURE 3: Time course for degradation of HT-29 cell total RNA by angiogenin and bovine pancreatic RNase. RNA was incubated with or without angiogenin or pancreatic RNase at 37 °C as described under Experimental Procedures. At the times indicated, formamide/formaldehyde reagent was added. Samples were then run on a 1.1% agarose gel under denaturing conditions. RNA was visualized with ethidium bromide. (Lanes 1–5) Samples containing 1.9 μM angiogenin incubated for 3, 10, 25, 60, and 255 min, respectively; (lane 6) control RNA sample incubated for 255 min; (lanes 7 and 8) samples containing 0.13 nM pancreatic RNase incubated for 3 and 30 min, respectively.

HPLC. Fractions were lyophilized and reconstituted with water, and aliquots were added to HT-29 cell RNA and incubated as above. Comigration of RNA-degrading activity and angiogenin was clearly evident (data not shown). The nature of this activity was investigated further by using a higher concentration of angiogenin (1.9 μM) and several different incubation times (Figure 3). The major degradation products formed after 60 min (lane 5) are apparently 100–500 nucleotides long and are not degraded substantially when the incubation time is increased to 255 min (lane 6). In contrast, under the same conditions pancreatic RNase (lanes 7 and 8) eventually generates small products that migrate ahead of the dye front.

Preliminary chemical modification experiments were performed examining the effects on angiogenin of two reagents known to inactivate bovine pancreatic RNase—bromoacetate (Barnard & Stein, 1959) and diethyl pyrocarbonate (Rosén & Fedorcsák, 1966). Both 30 mM bromoacetate (pH 5.5, 18 h at 25 °C) and 2 mM diethyl pyrocarbonate (pH 7.5, 10 min at 25 °C, followed by 0.5 M hydroxylamine for 30 min) eliminated more than 95% of the ribonucleolytic activity of angiogenin. This finding suggests the presence of critical histidine and lysine residues, as in pancreatic RNase.

A detailed examination of the kinetics and specificity of the action of angiogenin on RNA is in progress together with structural studies to define their relationships. Until recently, the rate and progress of this investigation has been dictated by the availability of sufficient quantities of angiogenin.

Discussion

The amino acid sequence of human tumor derived angiogenin (Fett et al., 1985) is remarkably homologous to that of the family of pancreatic RNases (Kurachi et al., 1985; Strydom et al., 1985). A separate communication will give a detailed analysis of this homology. Briefly, 43 (35%) of the 123 amino acids in angiogenin are identical with those at the corresponding positions in human pancreatic RNase (Beintema, 1984), and another 41 either are identical with residues in other pancreatic RNases (Blackburn & Moore, 1982; Bientema et al., 1985) or constitute conservative replacements resulting in an overall homology of 68%. Most importantly, virtually all of the active site components of bovine pancreatic RNase, as known from both chemical modifications (Stein & Barnard, 1959; Crestfield et al., 1963; Hirs et al., 1965) and several X-ray and neutron diffraction studies (Richards & Wyckoff, 1973; Wodak et al., 1977; Borkakoti, 1983; Wlodawer et al., 1983), are conserved. Table II lists these residues, their putative roles, and the corresponding amino acid residues in angiogenin. Four major segments of the primary structure are thought to constitute the catalytic and substrate-binding regions of RNase: they include the residues encompassing His-12, Lys-41, and His-119 and the loop formed by the disulfide bond between Cys-65 and Cys-72. His-12, Lys-41, and His-119—the three critical residues believed to participate in RNase catalysis—all have identical counterparts in angiogenin. The substrate-binding (i.e., specificity) residues Gln-11, Thr-45, Asn-71, Glu-111, and Ser-123 are also conserved. Asn-44 and Asp-121, whose roles are less well-defined, are present as well. The substitution of Ile for Val at position 43 is conservative, and this residue is not invariant among the pancreatic RNases of different species. In angiogenin a Leu replaces Phe-120 in RNase. However, a Phe → Leu substitution at this position has been shown to diminish activity toward C>p only by about 7-fold (Lin et al., 1972).

The Cys-65/Cys-72 disulfide bridge of RNase is thought to form a region that participates in binding the purine ring of inhibitors such as cytidyl(2′,5′)adenosine (and presumably the corresponding 3′,5′-dinucleotide substrate) (Wodak et al., 1977). Angiogenin lacks both of these cysteines and has two

Table II: Residues in the Active Site Region of RNase and Corresponding Amino Acids in Angiogenin

RNase residue[a]	proposed role(s)	angiogenin residue
His-12	removes 2′OH proton; H bonds to phosphate	His-13
His-119	protonates 5′O	His-114
Lys-41	stabilizes intermediate; H bonds to 2′OH	Lys-40
Gln-11	H bonds from side chain NH to phosphate	Gln-12
Val-43		Ile-42
Asn-44		Asn-43
Thr-45	H bonds from NH and OH to pyrimidine O2 and N3	Thr-44
Gln-69	H bonds from side chain C=O to purine N or O6	deleted
Asn-71	H bonds from side chain C=O to purine N or O6	Asn-68
Glu-111	H bonds from COO^- to purine N1	Glu-108
Phe-120	H bonds from amide NH to phosphate	Leu-115
Asp-121	H bonds from COO^- to His-119 Im	Asp-116
Ser-123	H bonds from OH to uracil O4	Ser-118

[a] The residues in the positions indicated are the same in both bovine and human pancreatic RNase. RNase residues and proposed roles listed were compiled from several X-ray and neutron diffraction studies of the interaction of bovine RNase A and RNase S with inhibitors (Richards & Wyckoff, 1973; Wodak et al., 1977; Borkakoti, 1983; Wlodawer et al., 1983). In some instances [e.g., Borkakoti (1983)], Lys-7 and Lys-66 have also been observed to be positioned in the active site region. While both of these residues are absent in angiogenin, it is known that neither is essential for RNase activity (Hofmann et al., 1971; Beintema et al., 1985).

fewer amino acids and only one identity in this region, which is highly conserved among the mammalian and marsupial pancreatic RNases. However, this region is completely different in turtle RNase which is fully functional but—like angiogenin—has deletions assigned to residues 69 and 70, does not have a Lys at position 66, and also lacks the disulfide bridge (Beintema et al., 1985).

A preliminary three-dimensional structure of angiogenin has been computed by energy minimization procedures, based on its homology to bovine pancreatic RNase (Palmer et al., 1986). The peptide backbones of the two proteins can be superimposed easily in the areas of regular structure. At this stage of the calculations no obvious differences are apparent that would preclude expression of some kind of pancreatic RNase-like activity by angiogenin.

In the light of all of these circumstances, the present investigation was undertaken to determine whether the structural homology between angiogenin and the pancreatic RNases relates to their both utilizing RNA as substrate, although the sites and/or nature of the cleavage might differ. Hence, we set out to determine whether or not angiogenin, in fact, exhibits *any* activity toward ribonucleic acids. Despite very sensitive assay methods and a variety of reaction conditions, no activity toward any of the pancreatic RNase substrates listed in Table I (top) could be detected.

Substrate specificity can of course vary markedly even among closely related enzymes. The serine proteases are among the best known examples of this phenomenon. Thus, the degree of sequence homology between trypsin, chymotrypsin, elastase, factor X, and kallikrein [see Dayhoff (1978)] is similar to that between angiogenin and pancreatic RNase. These proteases have the same triad of catalytic residues (Asp, His, Ser), yet each has a characteristic specificity. Accordingly, we considered the possibility that angiogenin might prefer substrates that are not attacked efficiently by the pancreatic RNases. Many such compounds were tested (Table I, bottom) but no activity could be detected.

To examine the possibility that the ribonucleolytic specificity of angiogenin might be very limited, its effects on 28S and 18S rRNA were determined by agarose gel electrophoresis. Endonucleolytic activity was clearly evident, proving that angiogenin indeed has the capacity to cleave RNA. However, unlike pancreatic RNase, it appears to generate relatively large fragments, containing from 100 to 500 nucleotides, suggesting only limited cleavage within each RNA species. The failure of angiogenin to degrade these fragments of rRNA further suggests either that they may be protected from angiogenin by virtue of their secondary structure or that its activity is highly specific. Alternatively, complete degradation may be prevented due to product inhibition. Such possibilities are under investigation.

The possibility had to be considered, of course, that the ribonucleolytic activity of angiogenin might be attributable to a contaminant rather than to the protein itself. The experimental results indicate that this is improbable. Thus, following extensive HPLC purification the activity comigrates with angiogenin. In addition, the most likely contaminants would be HT-RNase, followed by pancreatic and related RNases which are similar to angiogenin in charge and size. However, both pancreatic RNase and HT-RNase (not shown) degrade RNA to small fragments, in contrast to the limited digestion observed with angiogenin.

The results of chemical modification of angiogenin with bromoacetate and diethyl pyrocarbonate constitute compelling experimental verification of the relationship of its structure to its function. Bromoacetate alkylates His-119 and His-12 of bovine pancreatic RNase, and these derivatives are enzymatically inactive (Barnard & Stein, 1959; Heinrikson et al., 1965). Under very similar conditions bromoacetate destroys both the ribonucleolytic and the angiogenic activity of angiogenin virtually completely.

Modification of pancreatic RNase at Lys-41 also inactivates that enzyme. Following treatment with diethyl pyrocarbonate and hydroxylamine, angiogenin loses >95% of its ribonucleolytic activity, suggesting the presence of such a critical lysine in angiogenin.

While angiogenin clearly catalyzes the cleavage of ribosomal RNA, the precise features of the sites where cleavage occurs, optimum conditions of assay, and kinetic parameters are in the process of being established. So too are the potential functional implications of this enzymatic activity, particularly in regard to its physiological consequences and/or organogenic activity. It is possible, of course, that the nucleolytic activity of angiogenin is even more specific and selective toward a yet untested RNA substrate in a manner reminiscent of a restriction enzyme for DNA cleavage.

The cytotoxic protein α-sarcin may be a pertinent example to cite (Endo & Wool, 1982; Endo et al., 1983). It inhibits protein synthesis in eukaryotic cells by selectively catalyzing the cleavage of a single, unique phosphodiester bond in the 28S rRNA component of rat liver ribosomes or 60S ribosomal subunits. It has no effect on the 5S, 5.8S, and 18S RNA components, but when either total rRNA or naked 28S rRNA is the substrate, it can cause extensive progressive digestion. This endogenous nuclease activity of α-sarcin toward RNA is, however, very much less than that toward ribosomes. Such precedents call for guarded predictions regarding the mechanism by which angiogenin may exert its biological effects.

The sequence homology between angiogenin and the pancreatic RNases, which provided the incentive to search for ribonucleolytic activity, might be expected to hold additional clues to the mechanism of action of angiogenin. Current hypotheses (Folkman & Cotran, 1976; Vallee et al., 1985) relate the secretion of this protein by tumor cells to the induction of blood vessel formation which, in turn, promotes tumor growth, and a consequent primary role in tumor growth has been inferred from such suggestions. While the immediate target of angiogenin is unknown, these results indicate that RNA or a related molecule must at present be the likely candidate. Whether or not the process of angiogenesis entails a biochemical cascade and how extensive this might be are interesting questions that can now be approached experimentally.

A fascinating aspect of the RNase homology is the opportunity it provides to pursue the inhibition of angiogenin. In light of the above hypotheses the therapeutic potential of the many well-studied chemical modifications and various types of RNase inhibitors would be too great to be ignored. If this potential is verified, the many man-years of effort that have been dedicated to the study of the structure and function of the pancreatic ribonucleases might bear unexpected fruit in the form of an anti-angiogenic species that would impede or prevent the growth of tumors or of proliferative vascular disorders of various types.

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Registry No. RNase, 9001-99-4; angiogenin, 97950-81-7.

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