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CHARACTERIZATION OF RIBONUCLEOLYTIC ACTIVITY OF ANGIOGENIN TOWARDS tRNA

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Summary: Yeast tRNA is a convenient substrate for the assay of the ribonucleolytic activity of human angiogenin. The optimal pH, [NaCl], and temperature for tRNA cleavage by angiogenin are ~6.8, 15-30 mM, and ~55°C, respectively, as compared with ~8.0, 100-200 mM, and ~65°C, respectively, for RNase A. Polyanions and metals both inhibit angiogenin and RNase A but to different extents.

Angiogenin was identified on the basis of its potent blood vessel inducing properties (1) and found to be homologous to pancreatic RNases (2,3). Both proteins exhibit ribonucleolytic activities which differ, however, and are characteristic of each (4,5,6). Chemical modification (4), site-specific or regional mutagenesis (7,8), and placental ribonuclease inhibitor (9) can variously and differentially inhibit either the angiogenic or ribonucleolytic activities of angiogenin. This calls for the definition of these two different activities and their structural basis.

Angiogenin catalyzes the limited cleavage of ribosomal RNA either when free (4,6) or in the native, ribosome-bound state (5), and the patterns of cleavage differ from those produced by RNase A. A convenient, quantitative precipitation assay employing tRNA as a substrate (10) has allowed further characterization of this activity and its response to pH, salt concentration, temperature, and various inhibitors of RNase A.

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Abbreviations: RNase, pancreatic ribonuclease; RNase A, bovine pancreatic ribonuclease A; HSA, human serum albumin; IC_{50}, concentration at which 50% inhibition is observed.

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MATERIALS AND METHODS

Materials. Human angiogenin was isolated from either plasma (11) or conditioned media of baby hamster kidney cells genetically engineered to synthesize it (12) and subjected to a rigorous purification procedure in order to remove extraneous RNases (4,11). Angiogenin from either source exhibited the same ribonucleolytic activity. Angiogenin concentration was determined by amino acid analysis (Picotag, Waters Associates). RNase A (Code RAF) was purchased from Cooper Biomedical, and its concentration was determined spectrophotometrically using a molar absorptivity of 9800/M/cm at 278 nm (13). Metals (Specpure or Puratronic grade) were from Johnson Matthey Chemicals, heparin from Kabi, and poly(A)·poly(U) from P-L Biochemicals. Other polysaccharides and polynucleotides, all polyamino acids, tRNA (type X from yeast), and human serum albumin (HSA) were obtained from Sigma Chemical Co. Polynucleotide concentrations were determined spectrophotometrically using known molar absorptivities (14,15). Polyamino acid concentrations were determined by either amino acid analysis or spectrophotometric measurement of the tyrosine-containing polymers using a molar absorptivity of 1340/M/cm at 280 nm for tyrosine (16).

Assay. The enzymatic activities of angiogenin and RNase A were assayed by a modification of the procedure of Shapiro et al. (10). In a typical assay (100 μL), 1 μM angiogenin or 15 pM RNase A was incubated with 0.3% tRNA[1] in 30 mM Hepes, pH 6.8 (at 37°C), 30 mM NaCl, and HSA (0.001% or 0.01% for angiogenin or RNase A assays, respectively) for 2 h, 37°C. The reaction was terminated by chilling on ice and addition of an equal volume of 6% ice-cold perchloric acid. After 10 min on ice, the samples were centrifuged at 15,600 g for 10 min at 4°C, and the absorbance of a 1:3 dilution of the supernatant was measured at 260 nm. All assays were carried out in duplicate.

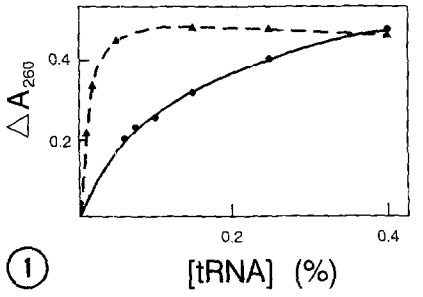
Absorbance readings of appropriate blanks were subtracted, and in some cases the resulting absorbance changes were normalized to the maximum change (typically ~0.5-1.0 unit) caused by the enzyme in a given experiment in order to give relative ΔA_{260} values. The standard curves for angiogenin were non-linear under the conditions employed in these experiments. Therefore, in experiments examining inhibition of the enzymatic activity of angiogenin, standard curves of absorbance changes caused by 0.32, 0.63, 0.95, 1.26, and 1.58 μM angiogenin were employed. The standard curves for RNase A were linear under the conditions used in these experiments.

RESULTS AND DISCUSSION

The ribonucleolytic activity of angiogenin has potential relevance to its angiogenic activity and therefore requires characterization (4,7). We have examined its activity towards tRNA and compared it with that of RNase A [17, and references therein].

[tRNA] Dependence. To yield comparable amounts of acid-soluble products requires from 10^4 to 10^5-fold more angiogenin than RNase A. Both of them catalyze the production of

[1] Similar absorbance changes were obtained when wheat germ RNA (Calbiochem) or highly polymerized yeast RNA (Calbiochem) were employed as substrates under these conditions.



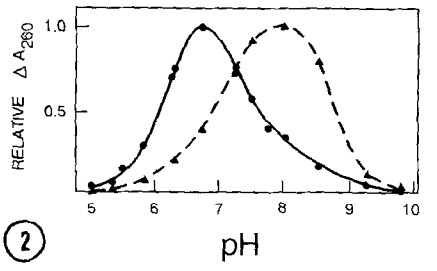


Figure 1: [tRNA] dependence of activities of 1 μM angiogenin (●—●) and 15 pM RNase A (▲--▲). Conditions were 30 mM Hepes, pH 7.3, 30 mM NaCl, 37°C, 2 h. All samples were adjusted to the same [tRNA] immediately before addition of perchloric acid.

Figure 2: pH dependence of activities of 1 μM angiogenin (●—●) and 15 pM RNase A (▲--▲) towards tRNA. Conditions were 30 mM acetate (pH 5, 5.5), Mes (5.3, 5.8, 6.3), Hepes (6.3, 6.8, 7.3, 7.8), Tris (7.5, 8.0, 8.5), or Ches (9.3, 9.8), 30 mM NaCl, 0.001-0.01% HSA, 0.3% tRNA, 37°C, 2 h. Relative ΔA_{260} values are defined in Materials and Methods. pH values stated here and elsewhere in text have been corrected for the temperature dependence of pK_a of the buffer using the following formula: $pH_{37°} = pH_{amb} + (\Delta pK_a/°C) \times \Delta°C$, where pH_{amb} is the pH at ambient temperature, $\Delta pK_a/°C$ is the temperature dependence of pK_a, and $\Delta°C$ is the difference between assay temperature (37°C) and ambient temperature.

increasing amounts of acid-soluble material as a function of substrate concentration (Figure 1). However, with RNase A the maximal velocity is reached at ~0.05% tRNA while that for angiogenin is still increasing at 0.4% tRNA. Lineweaver-Burk plots are non-linear for both.

pH and Salt Concentration Dependence. The pH profiles of both angiogenin and RNase A are bell-shaped in 30 mM NaCl (Figure 2). However, the pH optimum for angiogenin, ~6.8, is more than one pH unit lower than that for RNase A, ~8.0. Similarly, concentrations of low salt activate and those of high salt inhibit both angiogenin and RNase A, but both the optimum salt concentration and the magnitude of apparent activation are lower for angiogenin than for RNase A (Figure 3). For angiogenin, the optimal NaCl concentration at pH 6.8 is 15-30 mM and results in 115% of the activity in its absence (Figure 3, inset); this compares with values of 100-200 mM and ~500%, respectively, for RNase A. The activations obtained with Na_2SO_4 are much the same suggesting that they are primarily ionic strength effects (Figure 3). The concentration dependence of inhibition differs somewhat.

Temperature Dependence. The rate of acid-soluble product release increases as a function of temperature (Figure 4). For angiogenin the velocity is greatest at ~55°C, 10°C lower than that for RNase A, ~65°C. In part, this difference might reflect

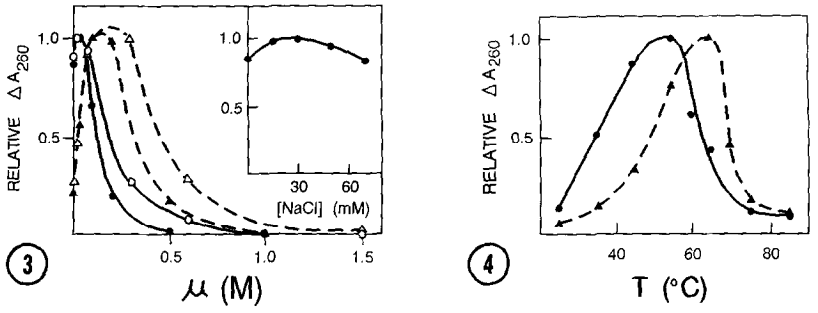


Figure 3: Dependence of activities of 1 μM angiogenin (—) and 15 pM RNase A (--) towards tRNA on ionic strength, μ, of added NaCl (●,▲) or Na_2SO_4 (○,△). Conditions were 30 mM Hepes, pH 6.8, 0.001-0.01% HSA, 0.3% tRNA, 37°C, 2 h. All samples were adjusted to the same salt concentration immediately before addition of perchloric acid. Relative ΔA_{260} values are defined in Materials and Methods. Inset: Dependence of activity of angiogenin towards tRNA on low [NaCl]. Conditions were as above.

Figure 4: Temperature dependence of activities of 1 μM angiogenin (●—●) and 15 pM RNase A (▲--▲) towards tRNA. Conditions were 30 mM Hepes, pH 6.8, 30 mM NaCl, 0.001-0.01% HSA, 0.3% tRNA, 20 min. Buffers were adjusted to appropriate pH at ambient temperature to give pH value of 6.8 at the experimental temperature according to the equation in legend to Figure 2. Relative ΔA_{260} values are defined in Materials and Methods.

the absence of the RNase disulfide bond Cys-65—Cys-72 in angiogenin (2) since disulfide bonds play a role in the thermal stability of proteins (18), which might be reflected in their activities.

Inhibitors. Placental ribonuclease inhibitor inhibits angiogenin (9,19), as is the case for a wide variety of RNase inhibitory polyanions and divalent cations [17, and references therein]. In general, molecules which inhibit RNase A inhibit

Table I. Inhibition of Angiogenin and RNase A by Polyanions and Cu^{2+} [a]

Inhibitor	IC_{50} [b]	
	Angiogenin	RNase A
poly(G)	140 μM	110 μM
poly(Glu,Tyr) (1:1)	40 μM	330 μM
heparin	700 μg/mL	500 μg/mL
Cu^{2+} [c]	30 μM	60 μM

[a] For poly(G), poly(Glu,Tyr), and heparin, conditions were 30 mM Na acetate, pH 5.5, 30 mM NaCl, 1.26 μM angiogenin or 30-60 pM RNase A, 0.001-0.01% HSA, 0.2% tRNA, 37°C, 10 h. For Cu^{2+}, conditions were 30 mM Hepes, pH 6.8, 30 mM NaCl, 1.26 μM angiogenin or 30 pM RNase A, 0.001-0.01% HSA, 0.2% tRNA, 37°C, 2 h. [b] For poly(G) and poly(Glu,Tyr), concentration is that of monomer. [c] Sulfate salt.

angiogenin, but to somewhat differing extents (Table I). For both enzymes the substrate concentration employed, 0.2%, is above the apparent K_m values; therefore, the actual K_i values are likely significantly lower than the IC_{50} values given here. In addition, since RNase A saturates at lower substrate concentrations than angiogenin (Figure 1), the differences between the K_i and IC_{50} values are likely to be greater for RNase A than for angiogenin.

Among various polynucleotides, including single and double stranded DNA from calf thymus and salmon sperm, respectively, poly(A)·poly(U), poly(A), poly(I), poly(U), and poly(G), the last was found to be the most potent inhibitor for both enzymes, with an IC_{50} value for angiogenin of 140 μM, slightly higher than that for RNase A, 110 μM. Poly(G), 2.5 mM, protects angiogenin almost completely from inactivation by either reductive methylation or modification by phenylglyoxal (10).

Several polyamino acids (random polymers, avg. MW ~26,000 - 43,000 Da), including poly(Glu,Tyr) (1:1 and 4:1), poly(Glu,Ala,Tyr) (1:1:1), poly(Asp), and poly(Glu), were also examined for inhibitory activity. Like for RNase A (20), the Glu,Tyr copolymer (1:1) was the most potent, with an IC_{50} value for angiogenin of 40 μM, several-fold lower than that for RNase A, 330 μM (Table I). Among various polysaccharides including chondroitin 6-sulfate, hyaluronic acid, and heparin, the last was the most potent inhibitor for both enzymes; it inhibits angiogenin and RNase A with IC_{50} values of 700 and 500 μg/mL, respectively.

Inhibition of angiogenin by Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}, all 1 mM, was also examined. Cu^{2+} and Zn^{2+} were the most effective ones, >99% and 93%, respectively. Cu^{2+} was suggested to inhibit RNase A by binding to the active site residues His-12 or His-119 or both (21). Since His-13 and His-114 likely occupy similar positions in the tertiary structure of angiogenin (22), Cu^{2+} might also be expected to inhibit angiogenin. It indeed does inhibits it with an IC_{50} value of 30 μM, slightly lower than that for RNase A, 60 μM.

The present characterization of the enzymatic activity of angiogenin towards tRNA confirms that it distinctly differs from that of RNase A in several respects. The physiologic relevance of pH, salt concentration, and various inhibitors, such as heparin or Cu^{++}, on this ribonucleolytic activity remains to be determined.

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